UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE FARMACIA

Departamento de Microbiología II

TESIS DOCTORAL

Functional characterization of the effector protein SteA of *Salmonella* Typhimurium through heterologous expression in *Saccharomyces cerevisiae*

Caracterización funcional de la proteína efectora SteA de *Salmonella* Typhimurium mediante expresión heteróloga en *Saccharomyces cerevisiae*

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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Directores

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Víctor Jiménez Cid

Madrid, 2018

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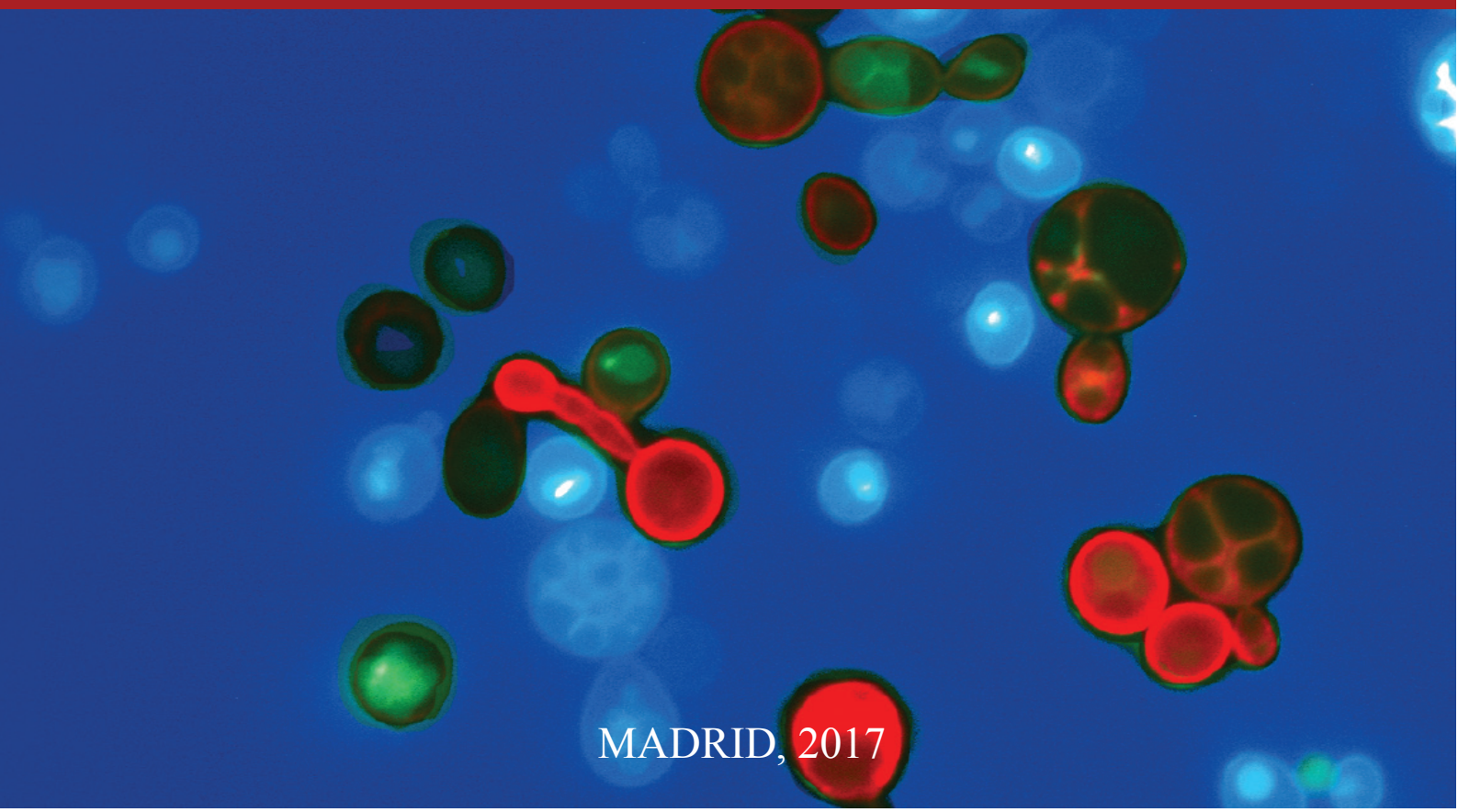

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Dra. MARÍA GLORIA MOLERO MARTÍN-PORTUGUÉS, Directora del departamento de Microbiología II de la facultad de Farmacia de la Universidad Complutense de Madrid

CERTIFICA:

Que **AHMAD ISMAIL** ha realizado en el departamento de Microbiología II de la Facultad de Farmacia de la Universidad Complutense de Madrid, bajo la dirección de los doctores **MARÍA MOLINA MARTÍN** y **VICTOR JIMÉNEZ CID**, el trabajo que presenta para optar al grado de Doctor en Farmacia con el título:

"Caracterización funcional de la proteína efectora SteA de *Salmonella* Typhimurium mediante expresión heteróloga en *Saccharomyces cerevisiae*"

Y para que así conste, firmo la presente certificación en Madrid, 2017

Fdo. María Gloria Molero Martín-Portugués

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To my family

To Cassiano

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ABBREVIATIONS LIST

ACBD3	Acyl-CoA binding domain-containing 3
ALP	Alkaline phosphatase
AP-3	Adaptor protein 3
CMAC	7-amino-4-chloromethylcoumarin
CORVET	Class C core vacuole/endosome tethering
CPS	Carboxypeptidase S
CPY	Carboxypeptidase Y
Cvt	Cytoplasm-to-vacuole targeting
DUB	Deubiquitinating enzymes
EEA1	Early endosome antigen 1
END	Endocytic pathway
ER	Endoplasmic reticulum
ERMES	Endoplasmic reticulum mitochondria encounter structure
ESCRT	Endosomal sorting complexes required for transport
FYCO1	FYVE and coiled-coil domain-containing protein 1
GEF	Guanine nucleotide exchange factor
GLUE	GRAM-Like ubiquitin binding in EAP45
GPCRs	G-protein coupled receptors
HOPS	Homotypic fusion and protein sorting
ILVs	Intralumenal vesicles
LAMP1	Late endosomal membrane protein 1
LBPA	Lysobisphosphatidic acid
LE	Late endosome
LPS	Lipopolysaccharides
MAMs	Mitochondria-ER associated membranes
MAPK	Mitogen-activated protein kinase
MARCH-8	Membrane-associated RING-CH
MCHII	Major histocompatibility complex class II
MMP	Mitochondrial membrane potential
MTOC	Microtubule-organizing center
MVB	Multivesicular body
PERP	p53-effector related to PMP-22
PKN1	PKC-related serine/threonine-protein kinase 1
PM	Plasma membrane
PMN	Polymorphonuclear
RCY	Recycling pathway
RILP	Rab-interacting lysosomal protein
ROS	Reactive oxygen species
SCAMP3	*Salmonella*-induced secretory carrier membrane protein 3
SCV	*Salmonella* containing vacuole

SEC	Secretory pathway
SIFs	*Salmonella*-induced filaments
SNX	Sorting nexins
SPI-1	*Salmonella* pathogenicity island-1
SPI-2	*Salmonella* pathogenicity island-2
T3SS	Type three secretion system
TGN	Trans-Golgi network
UBD	Ubiquiting-binding domain
UEV	Ubiquiting E2 variant
UIM	Ubiquitin-interacting motif
vATPase	Vacuolar ATPase
VPS	Vacuolar protein sorting

RESUMEN

Título: Caracterización funcional de la proteína efectora SteA de *Salmonella* Typhimurium mediante expresión heteróloga en *Saccharomyces cerevisiae*

Introducción

Salmonella enterica es un patógeno bacteriano intracelular facultativo que causa una amplia variedad de enfermedades en humanos (Figueira & Holden, 2012). La virulencia de *S. enterica* se basa en la translocación de dos conjuntos de efectores bacterianos en las células huésped a través de sistemas de secreción de tipo 3 (T3SS) codificados por las 2 islas de patogenicidad SPI-1 y SPI-2. Los efectores del T3SS de la SPI-1 permiten a las bacterias invadir la célula huésped, mientras que los efectores del T3SS de la SPI-2 son responsables de la replicación intracelular y supervivencia de *Salmonella* dentro de una vacuola llamada *Salmonella-containing vacuole* (SCV) (Figueira y Holden, 2012; LaRock *et al*., 2015). SteA está entre el grupo de efectores de *Salmonella* que pueden ser translocados tanto por el T3SS de la SPI-1 como el de laSPI-2 (Geddes *et al*., 2005; Cardenal-Muñoz y Ramos-Morales, 2011). Los mutantes carentes de este efector en *S.* Typhimurium muestran atenuación de la virulencia en modelos murinos de infección sistémica (Geddes *et al*., 2005, Lawley *et al*., 2006). Sin embargo, a pesar de su importancia para la patogénesis de *S.* Typhimurium, se han publicado pocos estudios sobre la función de SteA. Se ha descrito que SteA se localiza en la red trans-Golgi (TGN) cuando se expresa ectópicamente en células de mamífero no infectadas o se secreta por *S.* Typhimurium en células infectadas (Geddes *et al*., 2005). Sin embargo, en un estudio diferente, se demostró que el efector SteA translocado por la bacteria se localiza en los filamentos inducidos por *Salmonella* (SIFs) enriquecidos en el marcador del TGN 1,4-galactosiltransferasa (GalT) (Van Engelenburg & Palmer, 2010). Además, SteA contribuye al control de la dinámica de la membrana de la SCV y a la formación de SIFs, ya que las células infectadas con un mutante Δ*steA* muestran menos SIFs, aumento de la agrupación de SCVs y vacuolas morfológicamente anormales que contienen más de una bacteria (Domingues *et al*., 2014).

Desde hace más de cincuenta años se ha utilizado a la levadura *S. cerevisiae* como organismo modelo para el estudio de procesos celulares de células eucarióticas, como la transcripción y la traducción de ADN, el procesamiento de ARN, la

señalización celular, la regulación del citoesqueleto y el tráfico intracelular (Duina *et al*., 2014). Entre las posibles aplicaciones del modelo de levadura se encuentra la caracterización funcional de efectores bacterianos que son secretados por el T3SS para modificar los procesos celulares con el objetivo de permitir la supervivencia del patógeno y contribuir a la enfermedad (Popa *et al*., 2016). Este modelo permite el estudio de efectores de patógenos difíciles de cultivar o manipular, requiriendo sólo el ADN del patógeno en cuestión. La expresión de los efectores bacterianos en la levadura a menudo da lugar a una variedad de fenotipos o alteraciones del crecimiento, que pueden dar pistas sobre sus funciones en la célula hospedadora y su papel en la patogénesis, lo que puede ser confirmado por nuevas pruebas en levaduras u otros modelos celulares antes de realizar experimentos de infección en animales.

Objetivos

Nuestros objetivos están dirigidos a caracterizar funcionalmente la proteína efectora SteA de *Salmonella* Typhimurium en el modelo de levadura y son los siguientes:

- Caracterización de los efectos fenotípicos de la expresión de SteA en *S. cerevisiae*.
- Estudio de la localización subcelular de SteA en *S. cerevisiae* y sus determinantes.
- Identificación de las posibles dianas de SteA en el modelo de levadura.

Resultados y discusión

1. SteA causa inhibición del crecimiento y graves defectos en la morfología mitocondrial tras su expresión en células de levadura

La sobreexpresión de SteA en *S. cerevisiae* causó una inhibición significativa del crecimiento celular, acompañado de alteraciones en la morfología mitocondrial caracterizada por la aparición de mitocondrias condensadas. Este estudio ha permitido asignar un papel funcional al dominio amino-terminal de SteA, ya que los efectos observados eran dependientes de esta dicha región. Curiosamente, la lisina 36, dentro de un grupo de residuos básicos en la región amino-terminal de SteA, fue esencial para todos los efectos causados por SteA en las células de levadura. Uno de los complejos que mantienen la morfología tubular mitocondrial en la levadura es el complejo ERMES

(Mmm1, Mdm10, Mdm12 y Mdm34), localizado en la membrana externa de las mitocondrias y responsable del contacto de estos orgánulos con el retículo endoplásmico (RE). La mutación en cualquiera de estas proteínas conduce a una morfología mitocondrial anormal, pero con el citoesqueleto de actina intacto (Boldogh & Pon, 2006), lo que es similar al efecto que provoca SteA sobre las mitocondrias.

2. La capacidad de SteA de unirse a PI(4)P es un determinante de su localización subcelular dentro de las células de levadura

Nuestros resultados mostraron que SteA se localiza tanto en la membrana plasmática (MP) como en la vacuola cuando se expresa en levadura, y que los fosfoinositidos son marcadores diferenciales para la localización en dichas membranas celulares. Se encontró que la localización de SteA en la levadura era independiente del marcador principal de lamembrana plasmática, el PI (4,5) P_2. Además, en un mutante *pik1-ts* de *S. cerevisiae*, defectuoso en la PI 4-quinasa localizada en Golgi, SteA mantuvo intacta su localización. Sin embargo, la inactivación de la PI 4-quinasa Stt4 asociada con la membrana plasmática provocó la desaparición de SteA de dicha membrana, mientras que la localización vacuolar no se vio afectada. Estos resultados indican que la localización de la SteA en la membrana plasmática en células de levadura depende de su capacidad de unirse al fosfoinositido PI(4)P generado por Stt4. Como se ha descrito para SteA, otros efectores de *Salmonella* (SifA, SopD2, PipB2, SseF y SteC) se localizan tanto en los SIFs como en la membrana de la SCV en células de mamíferos (Schroeder *et al*., 2011), mientras que otros se localizan solo en la SCV, como SopE/E2 (Vonaesch *et al*., 2014) y SopB (Patel *et al*., 2009), pero ninguno de ellos se ha descrito que se una directamente a fosfoinositidos. Sin embargo, se sabe que efectores de otras bacterias explotan los fosfoinositidos de la células hospedadora para su orientación subcelular, tales como efectores de *Legionella* (LpnE, RidL, SetA, LidA, SidC y SidM), que se unen a PI(3)P y/o PI(4)P en la membrana de la *Legionella-containing vacuole* (LCV) (Weber *et al*., 2006; Ragaz *et al*., 2008; Brombacher *et al*., 2009; Weber *et al*., 2009[a]; Jank *et al*., 2012; Finsel *et al*. , 2013). Por lo tanto, la unión a fosfoinositidos no se había encontrado como un mecanismo de localización subcelular para ningún s efector de *Salmonella* antes de este trabajo. En este trabajo, hemos demostrado que la presencia de residuos básicos (región rica en lisina), y particularmente la lisina 36, en la

región N-terminal de SteA es esencial para su actividad de unión a PI(4)P y su localización tanto en la MPcomo en la vacuola en levadura. Esto sugiere que SteA también podría estar reconociendo este fosfoinositido en la membrana vacuolar. Se obtuvieron resultados similares de nuestro colaborador Dr. Mota en células de mamíferos, en los que PI(4)P fue necesario para la localización de SteA en la MP cuando se expresó ectópicamente en células HeLa, así como para su presencia en la membrana de la SCV y en los SIFs dentro de las células hospedadoras después de la infección por *S.* Typhimurium (Domingues *et al*., 2016).

Sorprendentemente, la sonda del PI(4)P, P4C, derivada del efector de *Legionella* SidC, no marcó la vacuola pero mostró más localización en la MP cuando se co-expresó con SteA que cuando se expresó sola, lo que sugiere que SteA podría estar regulando los niveles de PI(4)P en *S. cerevisiae*. Aunque se requieren estudios detallados adicionales sobre la estructura y la función de la actividad de unión a PI(4)P de SteA para entender los detalles estructurales de cómo se produce el reconocimiento de fosfoinositidos en este caso, nuestros resultados muestran que SteA y P4C podrían utilizarse para distinguir entre el PI(4)P generado en diferentes localizaciones subcelulares.

3. La vía VPS (*vacuolar protein sorting*) es necesaria para la localización de SteA en la vacuola de *S. cerevisiae*

En levadura, las proteínas son transportadas desde el retículo endoplásmico hasta el Golgi, donde se dirigen directamente hacia la vacuola a través de la vía de la fosfatasa alkalina (ALP) o a través de endosomas tempranos y endosomas tardíos/cuerpos multivesicular (MVB) a través de la vía VPS/CPY. Las proteínas derivadas de la membrana plasmática pueden ser internalizadas por endocitosis y transportadas a endosomas donde también pueden dirigirse a la vacuola a través de la vía VPS/CPY (Feyder *et al*., 2015). Nuestros resultados mostraron que SteA no se localiza en la vacuola en mutantes *vps* de la clase D y E, afectados en las proteínas de los complejos CORVET y ESCRT respectivamente. En su lugar, se acumuló en un compartimiento adyacente a la vacuola que co-localizó con el marcador de las endosomas tardios FM4-64 en los mutantes *vps* de clase E, o en puntos de diferente naturaleza en mutantes *vps*

de clase D. Estos resultados indican que SteA está dirigido a la vacuola a través del MVB.

4. La sobreexpresión de SteA muestra interacciones genéticas con mutaciones en genes relacionados con la homeostasis de las vacuolas

Además, nuestros resultados demostraron que la sobreexpresión de SteA mostró una interacción genética negativa con los mutantes *fab1Δ* y *vac14Δ* (defectuosos en los componentes del complejo proteico asociado a la vacuola que genera $PI(3,5)P_2$ en células de levadura), pero no se detectó ni una interacción física con dichos componentes ni la localización subcelular de SteA se vio afectada en estos mutantes. Este resultado sugiere que SteA está relacionado funcionalmente con el complejo Fab1, aunque la presencia de este complejo no es necesaria para dirigir SteA hacia la vacuola. Otros mutantes que mostraban una interacción genética negativa con SteA fueron *vps3*, *vps1* y *vps35*, mutantes que carecían de la quinasa PAK de Cla4, la subunidad de dineína (Dyn3) o proteínas relacionadas con el ensamblaje y la función de la v-ATPasa, como Vma1 o Vma21. Además, se encontró que la pérdida de Voa1, el factor de ensamblaje del sector V0 de la v-ATPasa, fue capaz de suprimir la toxicidad causada por la sobreexpresión de SteA. Todos estos hallazgos relacionados con la expresión de este efector de *Salmonella* en células de levadura, junto con la interacción específica de SteA con PI(4)P, indican que es probable que SteA interfiera con varios procesos relacionados con el tráfico de la membrana en células eucarióticas.

Conclusiones

1. SteA está altamente conservado entre diferentes serovares de *S. enterica*, y es específico para el género *Salmonella*.

2. La sobreexpresión de SteA en levadura provoca una inhibición significativa del crecimiento dependiente de su región amino-terminal.

3. La sobreexpresión de SteA conduce a defectos en la morfología mitocondrial, caracterizada por la aparición de mitocondrias condensadas. La región amino-terminal de SteA es necesaria y suficiente para causar este fenotipo mitocondrial.

4. SteA se localiza en la membrana plasmática y la vacuola cuando se expresa en *S. cerevisiae*, mientras que el fragmento amino-terminal correspondiente a los primeros 98 aminoácidos de SteA se localiza en el retículo endoplásmico.

5. La localización de la membrana plasmática de SteA requiere la presencia de PI(4)P generado por la quinasa Stt4 asociado a la membrana plasmática .

6. La unión de SteA a la membrana plasmática y la vacuola en *S. cerevisiae* requiere residuos básicos cerca de su extremo amino-terminal, específicamente la lisina 36. Este residuo básico también es necesario para inducir tanto la inhibición del crecimiento como la condensación mitocondrial en células de levadura.

7. Proteínas Vps de Clase D y E son necesarias para la localización vacuolar de SteA, lo que apunta a la importancia de la clasificación a nivel de los endosomas tardios/MVB en la orientación de SteA a la vacuola.

8. Los mutantes que carecen de las subunidades del complejo PI5-quinasa Vac14 y Fab1, muestran una interacción genética negativa con la sobreexpresión de SteA.

9. Las mutaciones en *CLA4*, *VPS1*, *VPS3* y *VPS35* causan un agravamiento fenotípico fenotípico de la inhibición del crecimiento inducida por SteA.

10. La ausencia de Voa1, una proteína del retículo endoplásmico que funciona en el ensamblaje del sector V0 de V-ATPasa, pero no de otras subunidades de V-ATPasa, suprime la inhibición del crecimiento inducida por SteA en *S. cerevisiae*.

SUMMARY

Title: Functional characterization of the effector protein SteA of *Salmonella* Typhimurium through heterologous expression in *Saccharomyces cerevisiae*

Introduction

Salmonella enterica serovars are facultative intracellular bacterial pathogens causing a wide variety of diseases in humans (Figueira & Holden, 2012). *S. enterica* virulence relies on secreting two sets of bacterial effectors into host cells through the SPI-1 and SPI-2 T3SSs. SPI-1 effectors enable bacteria to invade the host cell while SPI-2 effectors are responsible for their intracellular replication and survival within the *Salmonella*-containing vacuole (SCV) (Figueira & Holden, 2012; Ramos-Morales, 2012; LaRock *et al.*, 2015). SteA is among the group of *Salmonella* effectors that can be translocated by both the SPI-1 and the SPI-2 T3SSs (Geddes *et al.*, 2005; Cardenal-Munoz & Ramos-Morales, 2011), and mutants lacking this effector in *S. enterica* serovar Typhimurium display virulence attenuation in mouse models of systemic infection (Geddes *et al*., 2005; Lawley *et al*., 2006). However, in spite of its importance for *S.* Typhimurium pathogenesis, only few studies on SteA function have been published. It has been reported that SteA localizes to the *trans*-Golgi network (TGN) when ectopically expressed in uninfected mammalian cells or delivered by *S.* Typhimurium into infected host cells (Geddes *et al.*, 2005). However, in a different study, bacterially translocated SteA was shown to localize to *Salmonella*-induced tubules enriched in the TGN marker 1,4-galactosyltransferase (GalT) (Van Engelenburg & Palmer, 2010). In addition, SteA contributes to the control of SCV membrane dynamics and *Salmonella*-induced filaments (SIFs) formation, since cells infected with a Δ*steA* mutant showed less SIFs, increased clustering of SCVs, and morphologically abnormal vacuoles containing more than one bacterium (Domingues *et al.*, 2014).

For over fifty years *S. cerevisiae* has been used as a model organism for the study of cellular processes that occur in eukaryotic cells, such as transcription and translation of DNA, RNA processing, cell division, cell signaling, regulation of cytoskeleton and intracellular trafficking (Duina *et al*., 2014). Among the possible applications of the yeast model, is the functional characterization of bacterial protein effectors secreted by the T3SS to subvert cellular processes with the aim of supporting pathogen survival and contributing to disease (Popa *et al.*, 2016). This model system

allows the study of effectors from pathogens difficult to grow or manipulate, requiring only the DNA of the pathogen in question. The expression of bacterial effectors in yeast often results in a variety of robust phenotypes or growth alterations, which can generate hypotheses about their targets in the host cell and their role in pathogenesis, which can be further confirmed by additional tests in yeast or other cell models prior to costly infection experiments in animals.

Objectives

Our objectives aimed to functionally characterize the effector protein SteA of *Salmonella* Typhimurium in the yeast model and are the following:

- Characterization of the phenotypic effects of SteA expression in *S. cerevisiae.*
- Study of the subcellular localization of SteA in *S. cerevisiae* and its determinants.
- Identification of putative heterologous targets of SteA in the yeast model.

Results and discussion

1. SteA caused growth inhibition and defects in mitochondrial morphology upon expression in yeast cells

Overexpression of SteA in *S. cerevisiae* led to a significant inhibition of cell growth, accompanied by alterations in mitochondrial morphology characterized by the appearance of condensed mitochondria. This study allowed assigning a functional role to the amino-terminal domain of SteA, since the observed effects were dependent on this region. Interestingly, Lys36, within a cluster of basic residues at the amino-terminal region of SteA, was essential for all SteA effects observed in the yeast cell. One of the complexes which maintain mitochondrial tubular morphology in yeast, is the ERMES complex (Mmm1, Mdm10, Mdm12 and Mdm34), located at the mitochondria outer membrane and tethering these organelles to the ER. Mutations in any of these proteins are known to lead to an abnormal mitochondrial morphology but intact actin cytoskeleton (Boldogh & Pon, 2006), similar to the effect caused by SteA, suggesting that the ERMES function could be affected by SteA expression.

2. The ability of SteA to bind PI(4)P is a determinant of its subcellular localization within yeast cells

Our results showed that SteA localizes to both the plasma membrane (PM) and the vacuole when expressed in yeast, and phosphoinositide species are differential markers of cellular membranes. We found that SteA localization in yeast was independent on the main PM marker, PI(4,5)P_2. Also, in a *S. cerevisiae pik1*-ts mutant, defective in the Golgi-located PI 4-kinase, SteA kept its localization intact. However, the inactivation of the PM-associated PI 4-kinase Stt4 led to disappearance of the PM pool of SteA, whereas the vacuolar localization was not affected. These results indicate that localization of SteA at the PM in yeast cells is dependent on its ability to bind the PI(4)P phosphoinositide pools generated by Stt4. As shown for SteA, other *Salmonella* effectors (SifA, SopD2, PipB2, SseF, and SteC) localize both at the SIFs and SCV membrane in mammalian cells (Schroeder *et al*., 2011), while others localized just at the SCV, such as SopE/E2 (Vonaesch *et al*., 2014) and SopB (Patel *et al*., 2009), but none of them had been described to directly bind phosphoinositides. However, other bacterial effectors are known to exploit host cell phosphoinositides for subcellular targeting, such as *Legionella* effectors (LpnE, RidL, SetA, LidA, SidC and SidM), which bind to PI(3)P and/or PI(4)P at the LCV (*Legionella*-containing vacuole) membrane (Weber et al., 2006; Ragaz *et al*., 2008; Brombacher *et al*., 2009; Weber *et al*., 2009a; Jank *et al*., 2012; Finsel *et al*., 2013). Therefore, binding to phosphoinositides had not been previously recognized as a mechanism of subcellular targeting of *Salmonella* effectors. In this work, we have shown that the presence of basic residues (lysine-rich region), and particularly the K36, at the SteA N-terminal region was essential for its PI(4)P binding activity and its localization at both the PM and vacuole in yeast. This suggests that SteA might also be recognizing this phosphoinositide at the vacuolar membrane. Similar results were obtained from our collaborator Dr. Mota in mammalian cells, in which PI(4)P was necessary for localization of SteA at the PM when ectopically expressed in HeLa cells, as well as for its presence at the SCV membrane and SIFs within host cells upon *S*. Typhimurium infection (Domingues *et al*., 2016).

Surprisingly, the PI(4)P probe P4C, derived from the *Legionella* effector SidC, did not mark the vacuole but showed an enhanced localization at the PM when co-

expressed with SteA than when expressed alone, suggesting that SteA might be regulating PI(4)P levels in yeast. Although further detailed studies on structure and function of the PI(4)P binding activity of SteA are required to understand molecular details of how phosphoinositide recognition occurs, our results show that SteA and P4C have different preference for PI(4)P depending on its subcellular location and, therefore, these probes can be used to distinguish discrete PI(4)P cellular pools.

3. The vacuolar protein sorting pathway is required for localization of SteA at the vacuole in yeast

In yeast, proteins are transported from the ER to Golgi where they are sorted either directly toward the vacuole through the ALP pathway or through early endosomes and late endosomes/multivesicular bodies (MVB) via the VPS/CPY pathway. PM proteins can be internalized by endocytosis and transported to endosomes where they can also be sorted to the vacuole via the VPS/CPY pathway (Feyder *et al*., 2015). Our results showed that SteA fails to localize to the vacuole in both class D and E *vps* mutants, affected in proteins of the CORVET and ESCRT complexes, respectively. Instead, it accumulated either in a compartment adjacent to the vacuole that co-localized with the late endosomal marker FM4-64 in class E *vps* mutants, or in puncta of different nature in class D *vps* mutants. These results indicate that SteA is targeted to the vacuole via MVB sorting.

4. Overexpression of SteA shows genetic interactions with mutations in genes related to vacuole homeostasis

In addition, our results showed that SteA overexpression displayed a negative genetic interaction with both *fab1*Δ and *vac14*Δ mutants (defective in components of the vacuole-associate protein complex that generates PI(3,5)P_2 in yeast cells), but neither a physical interaction was detected nor the subcellular localization of SteA was affected in these mutants. This result suggests that SteA is functionally related to the Fab1 complex although the presence of this complex is not needed for SteA targeting toward the vacuole. Other mutants showing a negative genetic interaction with SteA were *vps3*, *vps1* and *vps35*, mutants lacking the PAK kinase Cla4, the dynein subunit Dyn3, or proteins related to v-ATPase assembly and function, like Vma1 or Vma21. Moreover, we found that loss of Voa1, the assembly factor of the v-ATPase V0 sector, was able to

suppress SteA toxicity. All these findings related to the expression of this *Salmonella* effector in yeast cells, together with the specific interaction of SteA with PI(4)P, indicate that SteA is likely interfering with several processes related to eukaryotic membrane trafficking.

Conclusions:

1. SteA is highly conserved among different *S. enterica* serovars, and it is specific for the *Salmonella* genus.

2. Overexpression of SteA in yeast provokes a significant growth inhibition dependent on its amino-terminal region.

3. SteA overexpression leads to defects in the mitochondrial morphology, characterized by the appearance of condensed mitochondria. The amino-terminal region of SteA is necessary and sufficient to cause this mitochondrial phenotype.

4. SteA localizes at the plasma membrane and vacuole when expressed in *S. cerevisiae*, whereas the amino-terminal fragment corresponding to the first 98 amino acids of SteA localizes to the ER.

5. The plasma membrane localization of SteA requires the presence of PI(4)P pools generated by the PM-associated PI 4-kinase Stt4.

6. Tethering SteA to the plasma membrane and vacuole in *S. cerevisiae* requires basic residues near its N-terminus, specifically Lys36. This basic residue is also necessary to induce both growth inhibition and mitochondrial condensation in yeast cells.

7. Class D and E Vps proteins are necessary for vacuolar localization of SteA, pointing to the importance of sorting at late endosome/MVB in SteA targeting to the vacuole.

8. Mutants lacking PI(3)P 5-kinase complex subunits Vac14 and Fab1, show a negative genetic interaction with SteA overexpression.

9. Mutations in *CLA4*, *VPS1*, *VPS3* and *VPS35* cause phenotypic enhancement of SteA-induced growth inhibition.

10. The absence of Voa1, an ER protein that functions in assembly of the V0 sector of V-ATPase, but not that of other V-ATPase subunits, suppresses SteA-induced growth inhibition in *S. cerevisiae*.

INTRODUCTION

I. THE GENUS *Salmonella*: MOLECULAR BASIS OF ITS PATHOGENICITY

I.1. *Salmonella*: generalities, taxonomy and nomenclature

Salmonella is a genus of Gram-negative facultative intracellular bacterial pathogens causing a wide variety of animal and human diseases ranging from mild enteritis to serious systemic infections such as typhoid fever (Schlumberger & Hardt, 2006; McGhie *et al.*, 2009; Figueira & Holden, 2012).

The genus *Salmonella* lies within the phylum *Proteobacteria*, class *Gammaproteobacteria*, order *Enterobacteriales* and family *Enterobacteriaceae*. *Salmonella* is divided into two species, *S. bongori* and *S. enterica*. There are 6 subspecies within *Salmonella enterica*: *salame*, *arizonae*, *diarizonae*, *houtenae*, *indica* and *enterica* (Heyndrickx *et al.*, 2005; Tindall *et al.*, 2005; Fàbrega & Vila, 2013). *Salmonella enterica* contains more than 2600 *serovars* based on their flagellar (H) and LPS (O) antigen structures (Gal-Mor *et al.*, 2014). Most *S. enterica* serovars associated with diseases in humans and other warm-blooded animals belong to the subspecies *enterica.* These serovars can be either host-adapted or non-host-adapted (Gal-Mor *et al.*, 2014). Host-adapted serovars cause life-threatening systemic disease in a limited number of related species, while non-host-adapted strains may cause gastroenteritis in many different host species.

Among the serovars considered as pathogens for the human being, it is important to distinguish between those causing typhoid or enteric fever, i. e. “typhoidal” serovars, from “non-typhoidal” serovars causing enteritis syndromes (Gal-Mor *et al.*, 2014).

The serovars causing enteric fever are *S.* Typhi and *S.* Paratyphi A, B, and C, which produce respectively typhoid fever and paratyphoid fever. They are pathogens that exclusively infect humans and are transmitted through ingestion of contaminated water or food. Enteric fever actually happens endemically in developing countries, being endemic in regions of Central Africa and Latin America and reaching high incidences in India and Southeastern Asia. It is associated with fever and abdominal symptoms, which appear after an incubation period that fluctuates between 3 and 21 days. After an initial phase with systemic symptoms such as diaphoresis, headache, dry

cough, myalgia and arthralgia, the fever appears. It can be accompanied with maculopapular rash and hepatic symptoms. The abdominal symptoms are common including pain and constipation (even more common than diarrhea). In some cases the disease turns to be chronic. The treatment of enteric fever involves hospitalization and intravenous or oral antibiotic therapy (Gal-Mor *et al.*, 2014).

Within the non-thypoidal serovars, there is a big variety of strains, included in the *Salmonella enterica* subspecies *enterica* serovars Typhimurium and Enteritidis (*S.* Typhimurium and *S.* Enteritidis), which fall in the non-host-adapted strains, and are thus able to cause diseases in a variety of host species. In humans, infection with either of these serovars results in a self-limiting gastroenteritis (salmonellosis) involving fever, diarrhea, and abdominal pain. Symptoms appear between 6 and 72 hours after the ingesta of contaminated food. In 5% of the cases a bacteremia can occur; it can also evolve into complications such as endocarditis, aneurism, osteomyelitis and reactive arthritis (Townes, 2010). The treatment consists of oral rehydration and nasogastric or intravenous rehydration in some occasions (Boyle *et al.*, 2007). These serovars are able to induce a systemic infection in mice, and young calves. In addition, they are also able to colonize poultry and adult cattle without symptoms (Zhang & Mosser, 2008, Boyle *et al.*, 2007).

I.2. *Salmonella* virulence: invasion of the intestinal epithelium

Salmonella invades both non-phagocytic and phagocytic cells. The analysis of *Salmonella* pathogenesis revealed the presence of two key virulence mechanisms: the interaction of *Salmonella* with epithelial cells and its survival and replication inside the phagocytes. The *Salmonella* pathogenicity island 1 (SPI-1) function is required for the former stage of *Salmonella* infection and the *Salmonella* pathogenicity island 2 (SPI-2) for the latter stage. The epithelial barrier can be crossed by bacteria either by passive transport facilitated by dendritic cells, extending pseudopods between epithelial cells, or by active invasion. As a gate of entry, *Salmonella* uses the epithelial M cells of the Peyer´s patches for translocation across the epithelial barrier. Once across the epithelial barrier, *Salmonella* is confronted by macrophages and can actively invade them or may be phagocyted (Fig. 1) (Hurley *et al.*, 2014).

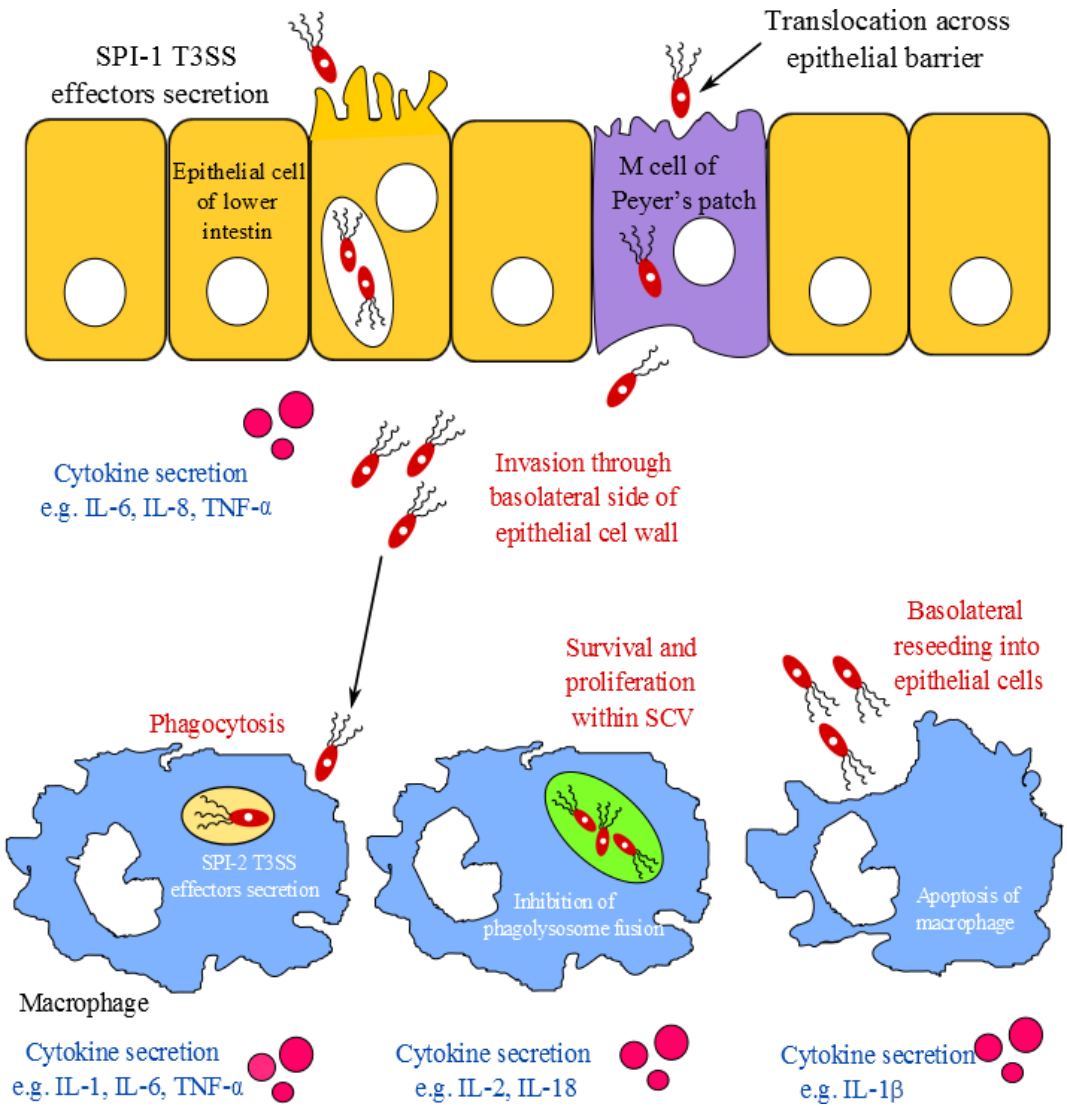

Figure 1. Schematic representation of host-pathogen interactions during pathogenesis of *Salmonella* infections. After oral ingestion, *Salmonella* cells reach the lower intestine, and then invasion of host epithelial cells can occur by translocation of *Salmonella* across epithelial barrier either using the epithelial M cells of the Peyer´s patches or via active invasion mediated by the secretion of SPI-1-encoded T3SS effector proteins. Afterwards, *Salmonella* is engulfed by the closest macrophages, where effector proteins of the SPI-2 are secreted in order to prevent fusion of the phagosome with the lysosome, and promote the survival and proliferation of *Salmonella* within SCV, resulting in cytokine secretion by the macrophage. Finally, the macrophages undergo apoptosis, and *Salmonella* escapes to re-invade epithelial cells or other phagocytic cells (adapted from Hurley *et al*., 2014).

Internalization of *Salmonella* in non-phagocytic cells of the intestinal epithelium occurs by a process that involves changes in the epithelial membrane such as cell microvilli degeneration, reorganization of the actin cytoskeleton and appearance of membrane extensions called ruffles that surround the bacterium. Thus, bacteria are

internalized and enclosed in a membrane-bound compartment called SCV (***S****almonella* **c**ontaining **v**acuole) (Ramsden *et al.*, 2007; Bakowski *et al.*, 2008; Steele-Mortimer, 2008). The SCV biogenesis can be divided in three phases (Fig. 2):

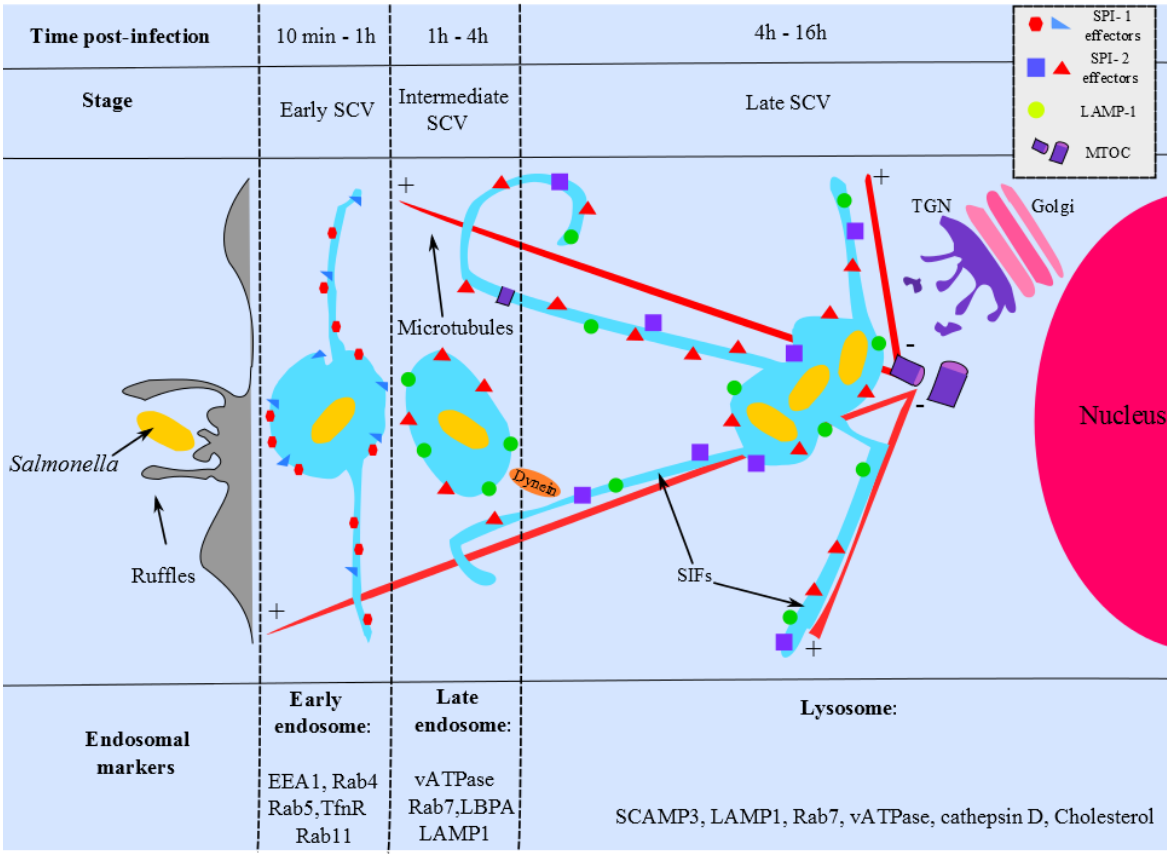

Figure 2. Schematic representation of the biogenesis of SCV. *Salmonella* internalization and the formation of the SCV start by the formation of ruffles at the host cell plasma membrane. During early SCV formation, the SCV acquires early endosomal markers, then those markers are replaced by late endosomal markers, leading to the formation of the intermediate SCV. Finally, *Salmonella* replicates and induces the formation of *Salmonella*-induced filaments (SIFs) along microtubules. MTOC stands for the microtubule-organizing center.

1) Early SCV (0-1 h post-infection): the vacuole acquires early endosome markers, such as early endosome antigen 1 (EEA1), GTPases Rab5 and Rab11, and the transferrin receptor TnfR (Ramsden *et al.*, 2007; Malik-Kale *et al.*, 2011; Schroeder *et al.*, 2011; Ramos-Morales, 2012).

2) Intermediate SCV (1-4 h post-infection): the markers of the early stage are immediately replaced with late endosomal markers, such as lysosomal associated membrane protein 1 (LAMP1), GTPase Rab7, lysosomal glycoproteins (lgps), and the vacuolar ATPase (vATPase), causing an

acidification of the lumen (Ramsden *et al.*, 2007; Malik-Kale *et al.*, 2011; Schroeder *et al.*, 2011; Ramos-Morales, 2012).

3) Late SCV (> 4 h post-infection): *Salmonella* replicates inside the SCV and induces the elongation of the vacuole into tubular structures called *Salmonella* induced filaments (SIFs), rich in LAMP1, vATPase and cathepsin D, that extend from the SCV along microtubules towards the cell periphery. Microcolonies appear, surrounded by Golgi membranes (Ramsden *et al.*, 2007; Malik-Kale *et al.*, 2011; Schroeder *et al.*, 2011; Ramos-Morales, 2012).

I.3. *Salmonella* pathogenicity islands and type III secretion system

The *Salmonella* Typhimurium genome has a size of 4,857 Kb (McClelland *et al.*, 2001). In addition, many strains contain a virulence plasmid of 94 Kb. The chromosome of *S.* Typhimurium contains 4,489 annotated CDS (coding sequences) and pseudogenes. A recent pangenomic approach on 12 *S.* Typhimurium strains recorded 3,192 genes in the core genome (Yue *et al.*, 2015). Among these, regions can be distinguished that are unique to certain strains, termed islands. Sometimes they encode for pathogenicity genes, and in such case they are called *Salmonella* pathogenicity islands (SPIs). These islands are found in regions adjacent to tRNA genes and have a G+C content different from the rest of the chromosome, which is 52% in the case of *Salmonella*, suggesting that they have been acquired by horizontal transmission. So far, 23 SPIs have been described and characterized, but only five of them (SPI-1 to SPI-5) are common to all serovars of *S. enterica* (Espinoza *et al.*, 2017).

The two most important pathogenicity islands of *Salmonella* are the SPI-1 and SPI-2, which encode type III secretion systems (T3SS) that enable bacteria either to invade eukaryotic cell or to replicate and survive inside a vacuole by injecting effector proteins into the cytoplasm through the plasma or vacuolar membrane respectively (Yip *et al.*, 2005; Galan & Wolf-Watz, 2006). The T3SS-1 apparatus or injectosome consists of two lower rings integrated into the plasma membrane (InvG), 40 nm diameter and 20 nm wide; two upper rings integrated in the outer membrane and peptidoglycan layer of the bacterium (PrgH) of 20 nm diameter and 18 nm wide united together through a shank (PrgK) and needle complex 50 nm long and 8 nm wide that protrudes from the

surface of the bacterium (PrgI) (Schraidt *et al.*, 2010) (Fig. 3). The injection process requires the presence of translocator proteins forming a pore in the host membrane. This injectosome has a common evolutionary origin with the bacterial flagellum, as their basal bodies are formed by proteins with conserved structures and both are able to export their own distal components (Diepold & Wagner, 2014).

The assembly of this structure occurs in several stages: first the proteins forming the base of the complex are secreted through the secretory pathway *sec*. Once in the periplasm, these proteins are associated with the inner membrane proteins forming a complex capable of exporting the components of the needle, whose length is controlled by another protein, InvJ (Diepold & Wagner, 2014). Once the complex of the needle is formed, the T3SS is able to export the corresponding effectors (Fig. 3). The T3SS is highly regulated at both transcriptional and post-transcriptional levels so that the effector proteins are secreted in a coordinated fashion in time and place (Galán, 2001).

The best-characterized *Salmonella* pathogenicity island is the SPI-1 (40 Kb), which contains genes that enable bacteria to invade non phagocytic cells. Unlike other SPIs, it is not located in an area adjacent to tRNA-encoding genes. The SPI-2 is a segment of 40 Kb adjacent to tRNA *valV* genes and contains 32 genes involved in the ability of developing systemic infection and intracellular survival. The SPI-1 appears in all subspecies of *Salmonella enterica*, while the SPI-2 does not, which indicates that it was probably acquired after the SPI-1, providing the ability to cause systemic infection (Marcus *et al*., 2000). The action of the main effectors secreted by these two T3SS of *Salmonella* is shown in figure 4.

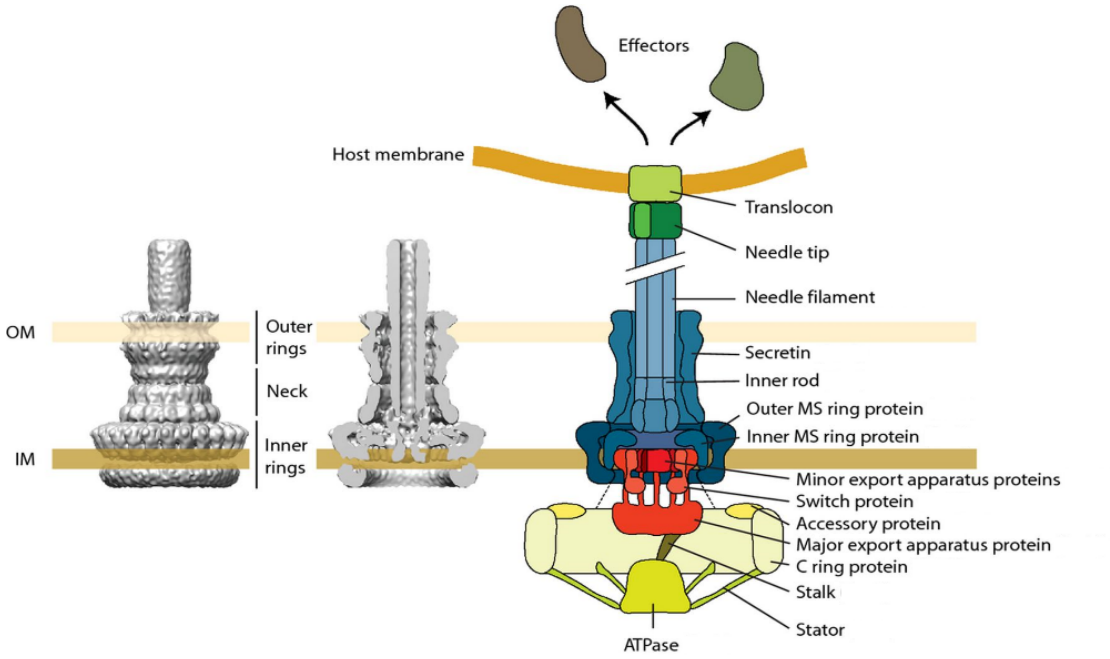

Figure 3. Schematic representation of the T3SS or injectosome. The left and middle panels show 3D representations of the injectosome and its components; inner membrane (IM), and outer membrane (OM). The right panel shows a drawing of the type III secretion system indicating all its components. Needle tip and translocated proteins in green, base and needle components in blue, cytoplasmic components are shaded in yellow, export apparatus components are depicted in red (Diepold & Wagner, 2014)**.**

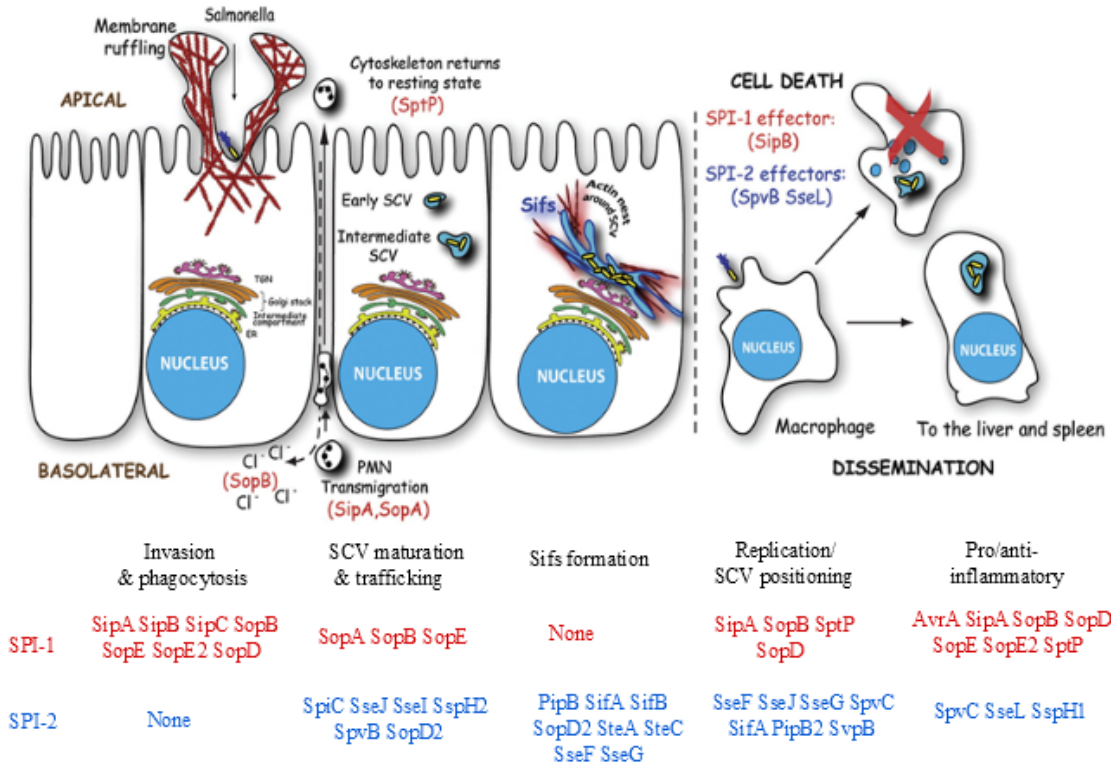

Figure 4. Schematic representation of the function of the main *Salmonella* T3SS effectors. *Salmonella* invade non-phagocytic cells by inducing membrane ruffling, enclosing bacteria in SCV, which traffic towards the perinuclear region of the host cell and mature via selective interactions with the endocytic pathway. Once the SCV is positioned next to the Golgi apparatus, intracellular bacterial replication begins. This stage is characterized by the formation of SIFs and the accumulation of F-actin around the bacterial phagosome (actin nest). Intestinal inflammation and diarrhea are caused by Cl^- secretion and polymorphonuclear leukocyte transmigration. *Salmonella* serovars associated with systemic disease are able to enter intestinal macrophages, inducing cell death as well as using them as a way to disseminate to the liver and spleen via the bloodstream and lymphatic system. SPI-1 and SPI-2 effectors involved in each individual infection stage are indicated (McGhie *et al.*, 2009).

I.3.1. Effectors of the *Salmonella* pathogenicity island 1 type III secretion system

The SPI-1 is located on the centisome 63 of *Salmonella*´s chromosome and contains approximately 40 genes encoding structure components of the T3SS-1, secreted effectors, their chaperones and transcriptional regulators (Que *et al.*, 2013). The SPI-1 T3SS is expressed in the intestinal lumen and its main function is to mediate entry of the bacteria into the host cells. SPI-1 is not required for systemic infection, so that mutants lacking this system have attenuated virulence in mice when orally inoculated, but are fully virulent after intraperitoneal inoculation (Groisman & Mouslim, 2000; Patel & Galan, 2005).

The T3SS SPI-1 secretes at least 19 polypeptides, including components of the secretion complex and the needle (Kimbrough & Miller, 2000). Other secreted proteins are effectors with various functions during the process of invasion (Haraga *et al*, 2008; Ramos-Morales, 2012) (Fig. 4 and Table I).

Table I. *Salmonella* pathogenicity island 1 type III secretion system effectors (* refers to the effectors translocated by both SPI-1 and SPI-2)

Effector	Function	Reference
SipA	-Actin polymerization -Ruffle formation -SCV maturation and positioning through cooperation with SifA	(Jepson *et al.*, 2001; Lilic *et al.*, 2003; Raffatellu *et al.*, 2005; Li *et al.*, 2013)
SipB	-Involved in autophagy -Induces apoptosis by activation of caspase-1 -Required for attachment to host epithelial cells	(Hersh & Zychlinsky, 1999; Hayward, 2000; Raffatellu *et al.*, 2005)
SipC	-Involved in actin bundling -Required for attachment to host epithelial cells -Regulates the trafficking of the host membrane protein PERP (p53-effector related to PMP-22)	(McGhie *et al.*, 2001; Myeni & Zhou, 2010; Hallstrom & McCormick, 2016)
SipD	-E3 ubiquitin ligase -Required for adhesion to host cells	(Lara-Tejero & Galan, 2009)
SlrP*	-Involved in cell death -Ubiquitination of Trx1, targets the human chaperone ERdj3	(Bernal-Bayard & Ramos-Morales, 2009; Bernal-Bayard *et al.*, 2010)
SopA	-E3 ubiquitin ligase -Phosphoinositide phosphatase -Migration of PMN -Mitochondrial alteration	(Layton *et al.*, 2005; Zhang *et al.*, 2006; Giacomodonato *et al.*, 2007)

Table II. Continued

Effector	Function	Reference
SopB/SigD	-Invasion of *Salmonella* -Actin polymerization during invasion -Induction of pro-inflammatory cytokines -Disruption of tight junctions -SCV maturation and positioning -Elimination of PIP_2 from the membrane -Recruiting of Rab5 and Vps34 to the SCV -Actin reorganization -Akt activation and inhibition of apoptosis -Modulates host cell exocytosis -Manipulates membrane surface charge and -trafficking of the SCV	(Marcus, 2002; Knodler *et al.*, 2005; Raffatellu *et al.*, 2005; Bakowski *et al.*, 2010; Perrett & Zhou, 2013)
SopE	-GEF of Cdc42, Rac1 and Rab5 -Induces inflammation -Disrupts the intestinal epithelial cell tight junctions -SCV maturation -Activation of caspase-1 in macrophages -Activation of Rab5, inhibiting the phagosome-lysosome fusion -Ruffles formation -Activates different sets of Rho GTPases -Contributes to the intracellular replication of *Salmonella*	(Friebel *et al.*, 2001; Deleu *et al.*, 2006; Humphreys *et al.*, 2012)
SopE2	-GEF of Cdc42 and Rac1 -Ruffles formation -Actin polymerization -Induces inflammation, by secretion of IL-8 -Disrupts the intestinal epithelia cell tight junctions	(Bakshi *et al.*, 2000; Friebel *et al.*, 2001; Raffatellu *et al.*, 2005; Giacomodonato *et al.*, 2007)
AvrA	-Acetyltransferase -Homolog toYopJ (*Yersinia*) -Inhibits apoptosis and inflammation -Stabilizes tight junctions -Mediates intracellular *Salmonella* survival -Inhibits NF-κB	(Collier-Hyams *et al.*, 2002; Liao *et al.*, 2008; Wu *et al.*, 2012)

Table I. Continued

Effector	Function	Reference
SptP	-Tyrosine phosphatase/GTPase activating protein -Promotes *Salmonella* intracellular replication -Downregulates of inflammation -Inhibits the mitogen-activated protein kinase (MAPK) pathway by inhibiting Raf activation -Binds and dephosphorylates the host AAA+ ATPase valosin-containing protein (VCP)	(Lin *et al.*, 2003; Humphreys *et al.*, 2009)

I.3.2. Effectors of the SPI-2 type III secretion system

The SPI-2 is located on the centisome 31 and was probably acquired subsequently to the SPI-1. Identification of proteins secreted by SPI-2 T3SS is conditioned by the fact that this system is not expressed in normal laboratory conditions, but only after entry of the bacteria into the host cell, at acid pH and low concentration of Mg^{2+}, Ca^{2+} and phosphate, and also requires the presence of the two components system SsrA/SsrB, regulated in turn by a second system, OmpR/EnvZ (Garmendia *et al.*, 2003; Kuhle & Hensel, 2004).

The T3SS SPI-2 is required for a later stage of infection (systemic spread), as well as for survival and intracellular replication (Figueira and Holden, 2012). It is also implicated in the induction of apoptosis in macrophages and allows *S.* Typhimurium to avoid NADPH oxidase-dependent killing by macrophages (Imke Hansen-Wester, 2001; Schlumberger & Hardt, 2006). Effectors secreted by the SPI2-T3SS are shown in Figure 4 and Table II.

Table II. *Salmonella* pathogenicity island 2 type III secretion system effectors (* refers to the effectors translocated by both SPI-1 and SPI-2)

Effector	Function	Reference
GogB	Unknown	(Coombes *et al.*, 2005; Pilar *et al.*, 2012)
GtgA	Unknown	(Ramos-Morales, 2012)
GtgB	Unknown	(Ramos-Morales, 2012)
GtgE*	-Protease	(Ramos-Morales, 2012)
PipB	-SIFs formation but not required for virulence	(Knodler *et al.*, 2004)
PipB2*	-SIFs formation -Recruits kinesin-1 to SCV -Reorganization of late endosomes and lysosomes to mediate SIFs extension	(Knodler & Steele-Mortimer, 2005; Henry *et al.*, 2006; Baison-Olmo *et al.*, 2012)
SopD*	-Contribute to virulence -Actin rearrangement during entry -SCV positioning -Membrane fission	(Jiang *et al.*, 2004; Raffatellu *et al.*, 2005; Bakowski *et al.*, 2007)
SopD2	-Involved in SIFs and SCV formation -Blocks delivery of endocytic cargo to lysosomes -Interacts with Rab7 by impairing its ability to bind RILP and FYCO1	(Brumell *et al.*, 2003; Schroeder *et al.*, 2010; D'Costa *et al.*, 2015)
SsaB (SpiC)	-Prevents fusion of late endosomes with SCV -Targets the mammalian Hook3 function to alter cellular traffic -Induces the secretion of IL-10 -Activation of MAPK in macrophages for the regulation of the expression of flagellin -Secretion of translocon proteins SseB and SseC	(Yu & Garvis, 2002; Freeman *et al.*, 2003; Uchiya & Nikai, 2008; Uchiya *et al.*, 2009)
SpvB	-Inhibits actin polymerization associated with SCV	(Otto, 2000;. Lesnick, 2001; Hochmann *et al.*, 2006)
SpvC*	-Downregulation of inflammation during intestinal phase of infection -Phosphothreonine lyase of MAPK proteins	(Mazurkiewicz *et al.*, 2008; Guiney & Fierer, 2011; Haneda *et al.*, 2012)

Table II. Continued

Effector	Function	Reference
SpvD*	-Prevent the activation of an NF-κB-dependent promoter -Causes nuclear accumulation of importin-α	(Rolhion *et al.*, 2016)
SrfH (SseI)	-Involved in macrophage motility -Important for early dissemination *Salmonella* to spleens in mice -Targets Filamin A, TRIP6, IQGAP1	(Worley *et al.*, 2006; McLaughlin *et al.*, 2009)
SseF	-SCV formation and positioning through recruitment of dynein, in harmony with SseG and SifA	(Deiwick *et al.*, 2006; Muller *et al.*, 2012; Ustun *et al.*, 2012)
SseG	-SCV formation and positioning	(Deiwick *et al.*, 2006)
SseJ	-Cholesterol acetyltransferase -Regulation of SCV membrane together with SifA -Promotes virulence by RhoA activation	(Ruiz-Albert, 2002; Lossi *et al.*, 2008; LaRock *et al.*, 2012)
SseL	-Modulates host´s inflammatory response by inhibiting NF-κB signaling activity and deubiquitination of IκBα	(Coombes *et al.*, 2007; Rytkonen *et al.*, 2007; Mesquita *et al.*, 2012)
SseK1	-Localized to host cell cytoplasm -Inhibits TFNα-induced NF-κB activation	(Kujat Choy *et al.*, 2004; Günster *et al.*, 2017)
SseK2	-Localized to host cell cytoplasm -Inhibits TFNα-induced NF-κB activation	(Kujat Choy *et al.*, 2004; Günster *et al.*, 2017)
SseK3	-Binds TRIM32 -Modulates host's NF-κB signaling activity -Inhibits TFNα-induced NF-κB activation	(Yang *et al.*, 2015; Günster *et al.*, 2017)
SifA	-GEF -Positioning of SCV -Formation and positioning of SIFs -Interaction with Rab7 impairing its ability to recruit RILP -Formation of tubular structure along with SseJ and RhoA -Reduction of recruitment of kinesin to the microtubules through interaction with SKIP, impairing its ability to bind with Rab9	(Brumell *et al.*, 2001; Brumell *et al.*, 2002; Boucrot *et al.*, 2003; Zhao *et al.*, 2015)

Table II. Continued

Effector	Function	Reference
SifB	-Localized on SCV and SIFs	(Freeman *et al.*, 2003)
SspH1*	-E3 ubiquitin ligase -Downregulates inflammatory response by inhibiting NF-κB signaling activity, Inhibition of IL-8 and ubiquitination of PKN1 (protein kinase N1)	(Haraga & Miller, 2006; Alexander *et al*., 2014)
SspH2	-E3 ubiquitin ligase -Interacts with actin-binding protein filamin -Exploits the NLR co-chaperone activity of SGT1 to subvert immunity	(Edward *et al*, 2003; Haraga & Miller, 2006)
SteA*	-Localized to the Golgi apparatus -Required for the colonization in mice model -Formation of SIFs -Control of membrane dynamics of *Salmonella* containing vacuoles	(Geddes *et al.*, 2005; Domingues *et al.*, 2014)
SteB*	-Required for the formation of biofilms -Required for full virulence	(Geddes *et al.*, 2005; Dong *et al*, 2008)
SteC	-Serine/threonine kinase -Localized to the SCV and SIFs -Formation of a F-actin network surrounding the SCV -Regulates the host actin cytoskeleton by targeting the MAPK MEK -Inhibits Cdc42-mediated signaling through binding to the exchange factor Cdc24	(Poh *et al.*, 2008; Fernandez-Pinar *et al.*, 2012; Odendall *et al.*, 2012)
SteD	-Mediates MARCH8-dependent ubiquitination of MHC II - Inhibits T Cell activation	(Bayer-Santos *et al*., 2016)
SteE*	Unknown	(Ramos-Morales, 2012)

Some of the effectors mentioned above (GtgE, PipB2, SpvC, SpvD, SspH1, SlrP, AvrA, SteA, SteB, and SteE) are secreted and translocated by both SPI-1 and SPI-2 T3SS.

I.4. Role of SPI-2 effectors in the interaction of *Salmonella* with the host cell

The SPI-2 T3SS is involved in many intracellular events. T3SS-2 secreted effectors function mainly in the maintenance and positioning of the SCV, whereas others act in the modulation of host immune signaling and/or target the host cytoskeleton (Fig. 5) (Holden & Figueira, 2012).

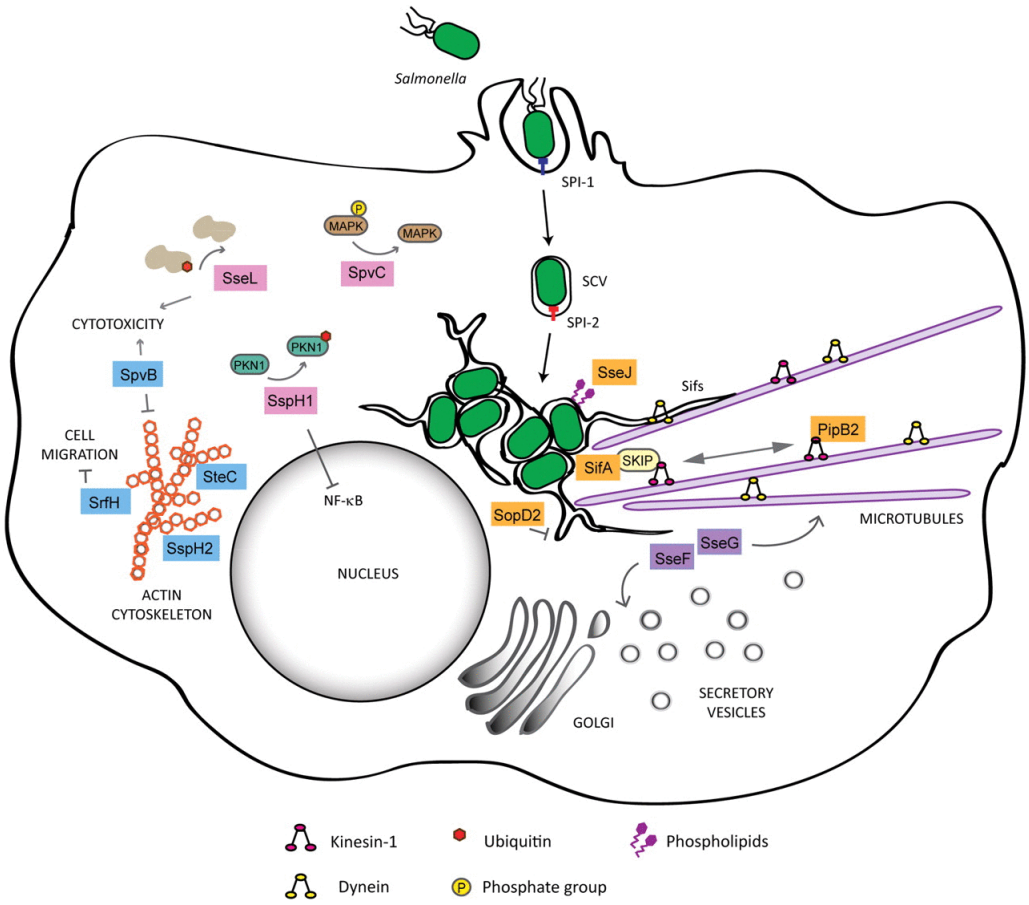

Figure 5. Function of the SPI-2 T3SS effectors and their involvement in the intracellular survival of *Salmonella*. Maintenance of the SCV is achieved through the action of SifA, SopD2, SseJ and PipB2 (orange boxes), and positioning near the Golgi apparatus by SseF and SseG (purple boxes). SteC, SspH2, SrfH and SpvB modulate the actin cytoskeleton (blue boxes). Other effectors target innate immune signaling (SspH1, SseL and SpvC; pink boxes) (taken from Holden & Figueira, 2012).

I.4.1. Maintenance and positioning of the SCV by SPI-2 effectors

Salmonella is able to modify the endocytic pathway to create a compartment suitable for its growth (Steele-Mortimer, 2008; Bakowski *et al*., 2008). The SPI-1 T3SS

effector SopB phosphatase is involved in both the formation and maturation of SCV (Perrett & Zhou, 2013). Then, SPI-2 T3SS delivery of effectors, like SifA, induces the formation of tubular structures called *Salmonella*-induced filaments (SIFs) that extend from SCVs throughout the host cell cytoplasm, supported by microtubules (Figs. 4 and 5) (Schroeder *et al*., 2011). SifA is involved in the formation of SIFs and the maintenance of SCV integrity through its interaction with GTPase Rab7 and SKIP (SifA and kinesin-interacting protein) (Malik-Kale *et al*., 2011; Zhao *et al*., 2015). In the absence of SifA or SKIP, an increased recruitment of kinesin to the SCV membrane occurs (Boucrot *et al*., 2005). This is mediated by SPI-2 T3SS effector, PipB2 that is involved in kinesin-driven SCV membrane dynamics and the radial extension of SIFs along microtubules (Knodler & Steele-Mortimer, 2005).

SopD2 localizes to the SIFs, SVC membranes, late endosomes and lysosomes. It is involved in SIFs formation and vacuolar membrane dynamics. (Figs. 4 and 5). In addition, SopD2 blocks delivery of endocytic cargoes to lysosomes and interacts with Rab7 by impairing its ability to bind its kinesin-binding effectors RILP and FYCO1 (Jiang *et al*., 2004; Schroeder *et al*., 2010; D'Costa *et al*., 2015).

Parallel to the formation of SIFs, the SCV moves from the cellular periphery to a juxtanuclear position at the microtubule organization center (MTOC), where Golgi stacks accumulate. Close contact between the SCV and the Golgi apparatus seems to play an important role in facilitating the acquisition of cellular components required for proliferation (Holden & Figueira, 2012; Yu *et al*., 2016). Bacterial division leads to the formation of microcolonies within the SCV through the action of both SPI-2 T3SS effectors SseF and SseG (Ramsden *et al*., 2007). SseF is involved in the formation of microtubule bundles and maintenance of the perinuclear position of SCV by recruiting dynein to SCV (Fig. 5) (Kuhle & Hensel., 2004; Abrahams *et al*., 2006). SseG is responsible for directing this positioning (Deiwick *et al*., 2006). Both SseF and SseG interact with mammalian protein ACBD3 (acyl-CoA binding domain containing 3) to anchor SCVs at the Golgi Network (Yu *et al*., 2016). Both effector proteins are considered to increase SIFs formation by modulating the aggregation of endosomal compatments, as s*seFΔ sseGΔ* mutant *Salmonella* induce less SIFs compared to wild type bacteria. On the other hand, other two SPI-2 T3SS effectors SseJ and SpvB antagonize SIFs formation. *Salmonella* mutants lacking either *sseJ* or *spvB* show an

increased number of SIFs (Ramsden *et al*., 2007). SseJ has phospholipase A and cholesterol acetyl transferase activities and modifies the membrane lipids of the SCV, thus facilitating fusion with other intracellular compartments (Lossi *et al*., 2008; Kolodziejek & Miller, 2015).

I.4.2. Modulation of the actin cytoskeleton SPI-2 T3SS effectors

At an early stage of infection SPI-1 effectors cause actin cytoskeleton rearrangements (Holden & Figueira, 2012; Raymond *et al*., 2013). Several hours later, *Salmonella* begins to replicate and induces the assembly of a dense F-actin meshwork in the proximity of SCVs in different cell types, including macrophages, epithelial cells and fibroblasts, in a SPI-2 T3SS-dependent manner (Holden & Figueira, 2012). SteC, which shows similarity to the human kinase Raf1, is the effector initiating this process, being its kinase activity necessary for actin meshwork formation (Fig. 5) (Poh *et al*., 2008). SteC has been also shown to inhibit Cdc42-mediated signaling through binding to the exchange factor Cdc24 when expressed in the yeast model *Saccharomyces cerevisiae* (Fernández- Piñar *et al.*, 2012).

SrfH and SspH2 localize as well to the SCV-associated F-actin meshwork, but do not appear to contribute to its formation. In fact, purified SspH2 leads to the inhibition of actin polymerization, suggesting that it might be counteracting the effect of SteC. SspH2 interacts with host cell actin regulators α-filamin and profilin-1 (Miao *et al*., 2003; Srikanth *et al*., 2011; Holden & Figueira, 2012).

Another effector that interferes with the actin cytoskeleton is SpvB, a protein encoded by the *spv* operon in the *Salmonella* virulence plasmid. It contributes to the alteration of actin cytoskeleton by ADP ribosylation of monomeric actin (Fig. 5) (Lesnick *et al*., 2011; Srikanth *et al*., 2011). A *spvB* mutant caused increased actin meshwork formation in HeLa cells (Miao et al., 2003), suggesting that the target of SpvB might be the F-actin meshwork formed as a result of the action of SteC (Holden & Figueira, 2012).

I.4.3. Modulation of host immune signaling by SPI-2 T3SS effectors

During infection, *Salmonella* is able to inhibit the inflammatory process in order to evade the immune response and promote its intracellular survival (Fig. 4). It modulates the expression of certain host cell genes by negatively regulating the NF-κB pathway through the ubiquitination of PKN1 (PKC-related serine/threonine-protein kinase) by SspH1, resulting in the inhibition the synthesis of the proinflammatory cytokine IL-8 (Fig. 5) (Haraga &Miller, 2006; Rohde *et al*., 2007). On the other hand, in infected macrophages there is an increase in the anti-inflammatory cytokine IL-10 dependent on the action of SpiC through the activation of the protein kinase-A (PKA) (Uchiya *et al*., 2004).

Another SPI-2 T3SS effector that modulates the immune signaling during infection is SpvC. This effector possesses a phosphothreonine lyase activity on MAPKs (Fig. 5) (Li *et al*., 2007). By inhibiting these pathways, SpvC exerts an anti-inflammatory effect; its overexpression causes the downregulation of the release of TNF-α and IL-8 from infected macrophages and epithelial cells, respectively (Mazurkiewicz *et al*., 2008; Haneda *et al*., 2012).

I.5. The *Salmonella* translocated effector SteA

SteA is among the group of *Salmonella* effectors that can be translocated by both the SPI-1 and the SPI-2 T3SSs (Geddes *et al.*, 2005; Cardenal-Muñoz & Ramos-Morales, 2011; Cardenal-Muñoz *et al*., 2014). It was first identified in a screening for new secreted effector proteins based on transposon mutagenesis coupled with the CyaA fusion method (Geddes et al., 2005). It was later isolated in a screen that aimed to unveil *Salmonella* Typhi genes that inhibited yeast growth when overexpressed (Alemán *et al*., 2009). Although SteA does not contribute directly to intracellular replication of *S.* Typhimurium (Figueira *et al.*, 2013, Domingues *et al.*, 2014), a Δ*steA* mutant shows virulence defects in mouse models of systemic and persistent infection (Geddes *et al.*, 2005, Lawley *et al.*, 2006). It has been also reported that SteA localizes to the *trans*-Golgi network (TGN), when ectopically expressed in uninfected mammalian cells or delivered by *S.* Typhimurium into infected host cells (Geddes *et al.*, 2005). However, in a different study, bacterially translocated SteA was shown to localize to *Salmonella*-induced tubules enriched in the TGN marker 1,4-galactosyltransferase (GalT) (Van

Engelenburg & Palmer, 2010). Amongst other *Salmonella* T3SS effectors, it has been recently shown by Domingues *et al* (2014) that SteA contributes to the control of SCV membrane dynamics and SIFs formation, since cells infected with Δ*steA* mutant *Salmonella* cells showed fewer SIFs and abnormal vacuoles (with more than one bacterium in each vacuole) with microcolonies displaying an excessive accumulation of LAMP1 that were frequently more compact than those formed by wild type bacteria (Domingues *et al.*, 2014). These authors also showed that SteA is functionally linked to SseF and SseG and suggested that it might contribute directly or indirectly to the regulation of microtubule motors on the SCV.

II. *S. cerevisiae* AS A HETEROLOGOUS MODEL FOR THE IDENTIFICATION AND CHARACTERIZATION OF BACTERIAL PROTEIN EFFECTORS

II.1 *S. cerevisiae* as a tool for the study of human proteins and drug screening

For over fifty years *S. cerevisiae* has been used as a model organism for the study of cellular processes that occur in eukaryotic cells, such as transcription and translation of DNA, RNA processing, cell signaling, regulation of cytoskeleton and intracellular trafficking (Duina *et al*., 2014). The high conservation of these processes in yeast, the ease of laboratory management and rapid growth, the relative simplicity of yeast genetics, and the existence of a wide variety of molecular tools, along with the fact that its genome sequence was published in 1996 (first sequenced eukaryotic genome) and more than 75% of its ORFs have known or predicted functions, have contributed to consider this microorganism as a key eukaryotic cell model. In addition, wide accessible information at a variety of web sites and databases on the molecular biology of *S. cerevisiae* and its applications as a cellular model is available (i.e. *Saccharomyces Genome Database* SGD). Around thirty percent of human genes known to be involved in diseases have orthologues in yeast, especially those that encode cell cycle regulators, components of signal transduction systems or metabolic routes. Due to the functional conservation from lower to higher eukaryotes, studies in yeast can provide much information on gene function in humans. For example, studies in yeast have contributed to the understanding of mechanisms regulating cell division and apoptosis whose alteration leads to cancer (Madeo *et al.*, 2002; Hartwell, 2002). In addition, the expression of human cDNAs in yeast allows functional complementation studies and identification of genes that are capable of interacting functionally with particular human cDNAs expressed in yeast. Actually, a systematic humanization of yeast by replacing over 400 essential yeast genes with their human orthologues revealed that about half of them could functionally replace their yeast counterparts (Kachroo *et al.,* 2015). Moreover, it is even possible the study of proteins implicated in human diseases that have no counterparts in yeast, but are capable of interfering with growth in this model (Tucker, 2002). For example, this is the case of humanized yeast systems

with some proteins implicated in oncogenesis, such as the module p53/Mdm2 (Di Ventura *et al.*, 2008), or the PI3K/PTEN/Akt pathway (Rodríguez-Escudero *et al.*, 2005; Andrés-Pons *et al*, 2007; Cid *et al.*, 2008; Rodríguez-Escudero *et al.*, 2009), and in neurodegenerative diseases, such as α-synuclein involved in Parkinson (Outeiro & Lindquist 2003; Auluck *et al*., 2010). Humanized yeast systems also served as platforms to develop high throughput screenings for the identification of chemical inhibitors of human proteins, such as drugs binding G-protein coupled receptors (GPCRs), involved in mental disorders, metabolic disorders, neurodegenerative diseases, cardiovascular diseases, and cancer (Overington *et al*., 2006; Schütz *et al*., 2016).

S. cerevisiae has also become one of the eukaryotic model choice systems for the development of genomic technology. Besides the application of the DNA microarray technology and proteomics, different genomic mutant collections have been obtained, both diploid and haploid, in which each strain carries a genetically engineered version of each of the approximately 6200 annotated ORFs, which can be used for large-scale phenotypic assays (Giaever *et al.*, 2002; Boone *et al*., 2007) Also, a genomic collection of GFP fusions has been developed for subcellular localization assays (Huh *et al*., 2003), as well as genomic collections of epitope-tagged proteins for expression and co-immunoprecipitation experiments, and fusions to the binding and activation domain of Gal4 for two-hybrid assays (Ghaemmaghami *et al.*, 2003).Whole genome deletion mutant collections also allow searching for synthetic lethality by combination with other mutations or the action of chemical compounds (Boone *et al*., 2007).

II.2 *S. cerevisiae* as a model system for the identification and characterization of bacterial virulence effectors

Among the possible applications of the yeast model, is the functional characterization of bacterial protein effectors secreted by the T3SS to subvert cellular processes with the aim of pathogen survival and contributing to disease (Rodríguez-Escudero *et al.*, 2005; Popa *et al.*, 2016). Such effector proteins must be expressed *de novo* in yeast because such secretory system is unable to inject proteins through the fungal cell wall. This model system allows the study of effectors from pathogens difficult to grow or manipulate, requiring only the DNA of the pathogen in question. The expression of bacterial effectors in yeast often results in a variety of robust

phenotypes or growth alterations, which can generate hypotheses about their role in pathogenesis, that can be further confirmed by new tests in yeast or other cell models prior to infection experiments in animals. The effect in yeast due to the expression of bacterial effector proteins is in many cases a very specific and sensitive indicator of the action of these proteins in the host cell. In a recent review on this topic, it is stated that, upon expression in yeast, T3SS bacterial effectors caused a perturbation of conserved cellular processes, with 84% of cases accompanied by growth inhibition. The most frequently observed effects are Rho GTPase signaling modulation, cytoskeleton disruption, MAPK signaling interference, cell death modulation, trafficking perturbation, and membrane alterations (Popa *et al.*, 2016).

Among virulence factors of pathogenic bacteria of both plants and animals that inhibited growth when expressed in yeast, are found effectors secreted by *Pseudomonas syringae* (Salomon *et al.*, 2012), *Pseudomonas aeruginosa* (Stirling & Evans, 2006; Burstein *et al.*, 2015), *Shigella flexneri* (Slagowski *et al.*, 2008) , *Legionella pneumophila* (Campodonico *et al.,* 2005; Derre & Isberg, 2005; O'Brien *et al.,* 2015) , *Chlamydia trachomatis (*Sisko *et al.,* 2006*)*, *Escherichia coli* (Rodríguez-Escudero *et al.*, 2005a; Popa *et al.*, 2016), *Coxiella burnetti* (Rodríguez-Escudero *et al*., 2016) and *Yersinia enterocolitica* (Nejedlik *et al.*, 2004). In the particular case of *Salmonella,* several effectors have been described to have an inhibitory effect on yeast growth, and our research group has studied some of them. Table III summarizes the list of *Salmonella* T3SS bacterial effectors studied in *S. cerevisiae* and the specific phenotypes that their expression causes on yeast cells. The versatility of yeast and its compliance for high-throughput functional studies have contributed to the prosperity and the success of this heterologous system that, in recent years, has been used to study more than 100 effectors (Siggers & Lesser, 2008; Curak *et al.*, 2009; Popa *et al.*, 2016).

A very important factor to consider in relation to growth inhibition is the strength of the promoter used in the heterologous expression. Expression of bacterial virulence factors in yeast is usually done under the control of an inducible promoter in order to control the possible growth inhibitory effect. To this end, the strong promoter of the *GAL1* gene, inducible by galactose (Rodríguez-Pachón *et al.*, 2002), or the weaker promoter of the *CUP1* gene, controlled by the presence copper (Mao *et al.*, 2002; Peng *et al.*, 2015) are often used. As for the number of copies, either centromeric

(1-3 copies) or 2 micron-derived plasmids (40-60 copies) can be used, but also a single copy can be integrated in the yeast genome by direct homologous recombination (Siggers & Lesser, 2008). Moreover, the fusion of the protein effector to an epitope such as GST or GFP may also influence its inhibitory effect. An example is Map of EPEC; its amino-terminal GST fusion inhibits growth while the carboxy-terminal GFP fusion does not (Rodríguez-Escudero *et al.*, 2005).

To monitor *S. cerevisiae* growth inhibition, there are many options such as optical density measurement in liquid medium, measurement of the cellular respiration using colorants, and the most used one is the serial dilution spot assay in solid medium (Sisko *et al.*, 2006). In addition, yeast can be used to check subcellular localization patterns, using GFP-tagged proteins and fluorescence microscopy (Huh *et al.*, 2003), as well as to study the alterations in the morphology of cytoskeleton and organelles, endocytosis and vesicle trafficking or signal transduction pathways caused by the expression of the bacterial protein (Siggers & Lesser, 2008).

Moreover, a variety of available yeast proteomic and genomic tools can be also used to identify and characterize bacterial effectors. Interacting proteins, effector targets, and transcriptional response may be monitored using techniques such as yeast two-hybrid, multi-copy suppression profiling, and transcriptomics. Recently, yeast genetics have been thoroughly exploited in order to check effector-effector interactions by screening all known 300 *Legionella pneumophila* effector proteins using a SGA (synthetic genetic array) screen in *S. cerevisiae* (Urbanus *et al.*, 2016). When the heterologous expression of the effector causes yeast growth inhibition, the target of the effector can be identified using the PGA (pathogenic genetic array) technique, that allows the transformation of the vector expressing the effector in the whole yeast mutant collection to identify suppressors (Curak *et al.*, 2009). Figure 6 shows a summary of functional assays and genomic tools used to study bacterial effectors in yeast.

Table III. List of *S*. Typhimurium effectors studied in *S. cerevisiae*

Effector	Phenotypes in yeast	Reference
AvrA	Growth inhibition and inhibits the high osmolarity glycerol (HOG) MAPK pathway	(Du & Galan, 2009)
SopE2	Growth inhibition, activation of MAPK signaling	(Rodríguez-Pachón *et al.*, 2002)
SptP	Growth inhibition, inhibition of MAPK signaling	(Rodríguez-Pachón *et al.*, 2002)
SopB	Growth inhibition, actin depolarization, disruption of cytoskeleton, inhibition of MAPK signaling, interaction with yeast Cdc42	(Alemán *et al.*, 2005; Rodríguez-Escudero *et al.*, 2006; Rodríguez-Escudero *et al.*, 2011)
SteC	Growth inhibition, disruption of actin cytoskeleton, inhibition of MAPK signaling, interaction with GEF Cdc24	(Aleman *et al.*, 2009; Fernandez-Pinar *et al.*, 2012)
SseF	Growth inhibition	(Aleman *et al.*, 2009)
SspH2	No alteration of cell cycle or effect on cell viability	(Bhavsar *et al.*, 2013)
SspA	Growth inhibition, disruption of actin cytoskeleton	(Lesser & Miller, 2001)
SifA	Changes yeast peroxisome abundance and morphology by altering Rho1 activity	(Vinh *et al.*, 2010)

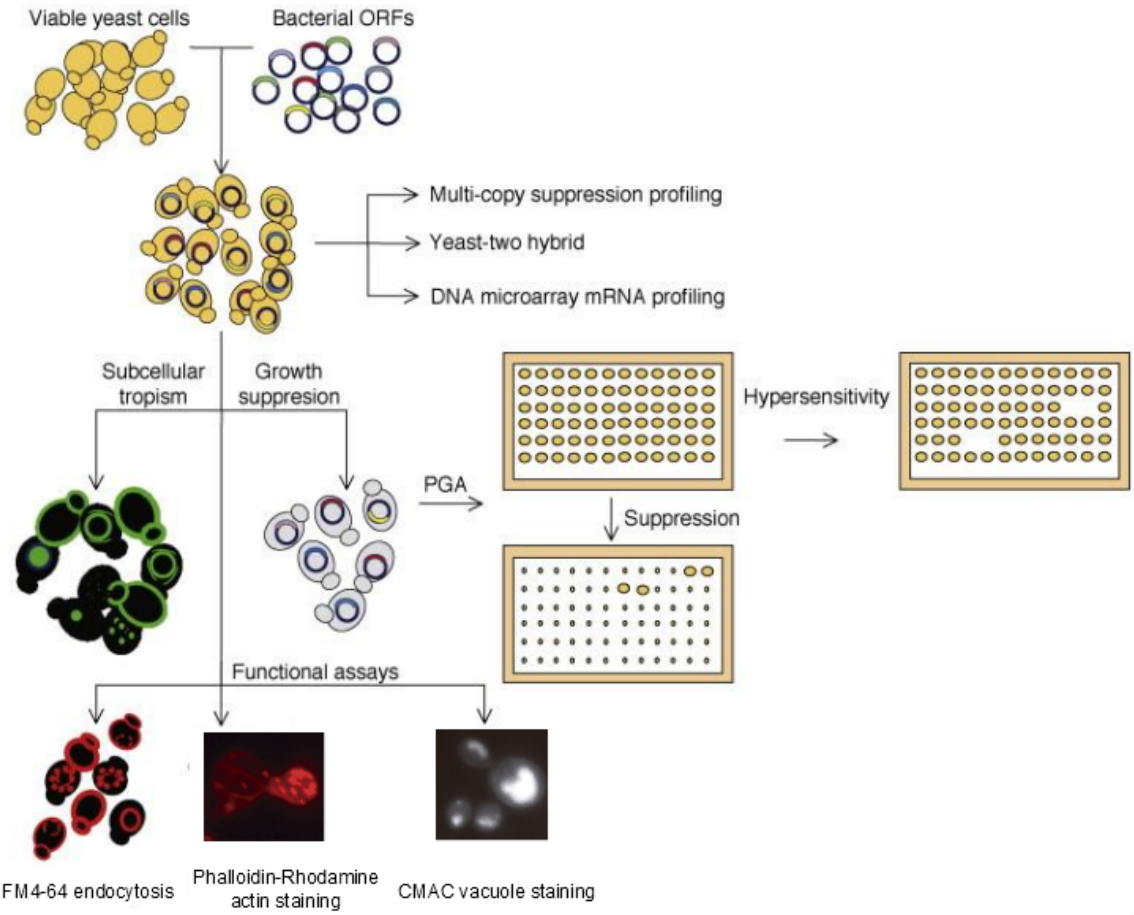

Figure 6. *S. cerevisiae* functional assays and genomic tools used to study bacterial effectors. Bacterial ORFs may be studied through expression in yeast using the indicated methodologies. (adapted from Curak *et al.*, 2009)

III. INTRACELLULAR MEMBRANE TRAFFICKING IN YEAST

III.1 Membrane trafficking in yeast: generalities

In yeast cells, both soluble and transmembrane proteins are generally produced at or transferred to the endoplasmic reticulum (ER), and afterwards transported to their precise target compartments in order to accomplish their function, undergo modification, or be degraded. After being translocated to the ER, newly synthesized proteins are directed to the Golgi through COP-II (anterograde transport), and some proteins are recycled or transported back to the ER from the Golgi through COP-I vesicles (retrograde transport). From the Golgi, some of them will be secreted through the secretory (SEC) pathway, or transported to the PM. Others will be targeted directly to the vacuole through the alkaline phosphatase (ALP) pathway or through endosomes via the carboxypeptidase Y/vacuolar protein sorting (CPY/VPS) pathway. PM-derived proteins can be internalized by endocytosis (END pathway) and translocated to the early endosome (EE) where they are sorted; some proteins undergoing sorting towards the vacuole and others being redirected to the trans-Golgi network (TGN) via the recycling pathway (RCY), where they are readdressed to the PM (Feyder *et al.*, 2015). Figure 7 summarizes the membrane trafficking pathways in yeast.

III.1.1 Endocytosis

In eukaryotic cells, the endocytic pathway (END) allows proteins from the extracellular medium and plasma membrane to be internalized and delivered either to the vacuole for degradation or to the TGN for recycling (Fig. 7) (Conibear, 2010). Two main types of endocytosis are known: receptor mediated endocytosis, triggered after the binding of the ligand by its receptor, and fluid-phase endocytosis during which vesicles sequester extra-cellular material non-specifically (Feyder *et al.*, 2015).

In *S. cerevisiae*, endocytosis consists of 3 main stages: Endocytic site initiation, invagination and scission, and finally release. The initiation site is formed by the recruitment of clathrin via AP-2 adaptor complex, leading to the formation of the early coat where cargo proteins are clustered. The initiation is finished by the formation of a late coat through recruitment of ENTH (epsin N-terminal homology) and ANTH (AP180 N-terminal homology) domains-containing proteins named epsins, Ent1/Ent2,

and Sla2. Afterwards the WASP (Wiskott-Aldrich syndrome family protein)/myosin module is recruited, leading to actin polymerization and plasma membrane invagination. Once the invagination is completed, the fission module starts releasing vesicles into the cytoplasm (Feyder *et al.*, 2015).

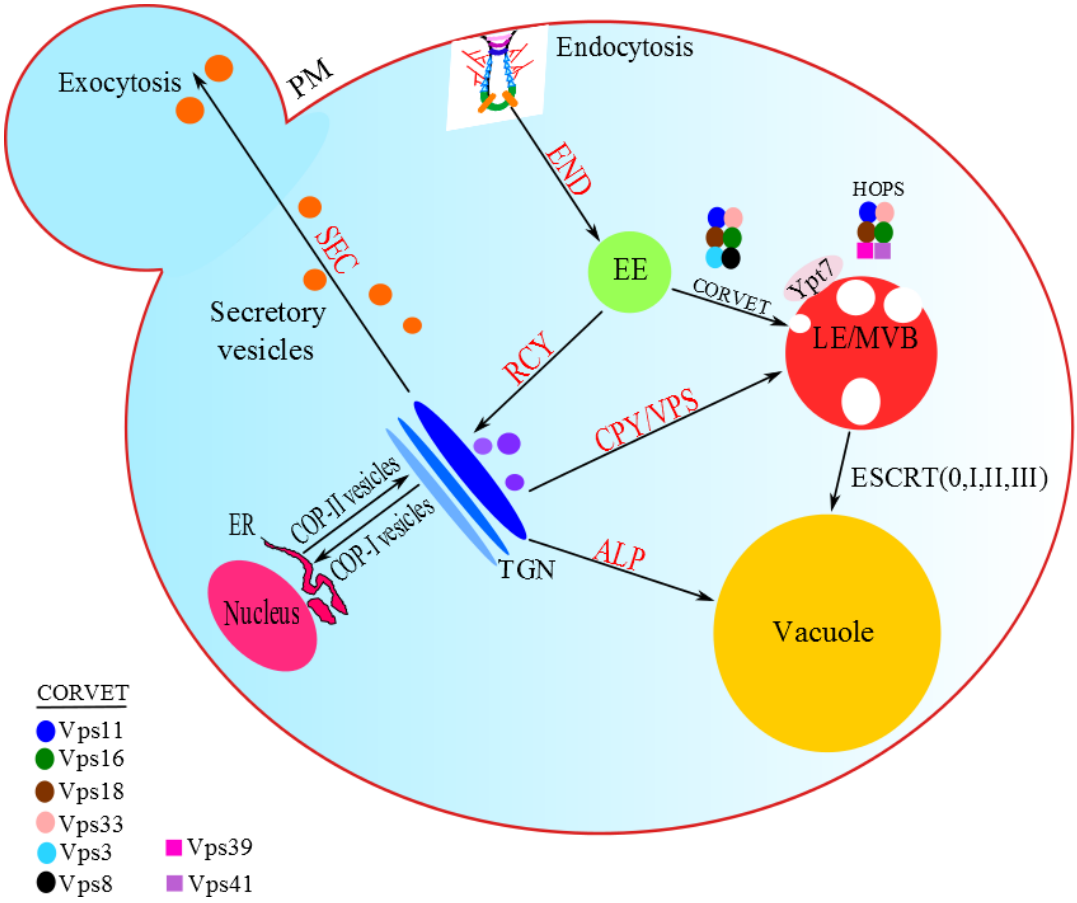

Figure 7. Intracellular membrane trafficking pathways in yeast. Golgi derived proteins can be either secreted (SEC pathway), or transported to the vacuole following two main pathways. Proteins that follow the CPY (carboxypeptidase Y) pathway are sorted through clathrin-coated Golgi vesicles and reach the vacuole by passing the endosomal/MVB compartments. Proteins that follow the ALP (alkaline phosphatase) pathway are sorted directly to the vacuole through different type of Golgi derived vesicles. CPY pathway and END pathway converge at the MVB (multivesicular body). At the early endosomes (EE), proteins are sorted either to the late endosome (LE)/MVB, or to the Golgi to avoid degradation through the recycling pathway (RCY).

The END pathway starts by the formation of the early endosomes (EE), fed by endocytic vesicles. Proteins undergoing recycling are directed from EE to the TGN via the recycling pathway (RCY) (Fig. 7), from where they can be secreted via the

exocytosis again to the plasma membrane. Early endosomes mature and are subsequently are converted to late endosomes, also known as multivesicular bodies (MVB) or pre-vacuolar compartments (Clague & Urbé, 2008), which control traffic corresponding to cargos that either are essential for proper vacuolar function or should be degraded in the vacuole. The MVB fuses with the vacuole, the terminal compartment of the endocytic pathway in yeast cells, and liberates its content into the vacuolar lumen (Fig. 7).

III.1.2 Traffic from the Golgi to the vacuole via the VPS/CPY pathway

Many proteins are targeted to the vacuole through the VPS/CPY pathway. The *VPS* genes were identified in a set of genetic screens, generating 61 mutants defective in vacuolar protein sorting. The hallmark of all *vps* mutants is the mis-sorting of CPY, and they are classified in 6 classes (A-F) based on the morphology of their vacuoles. Class A *vps* mutant cells show normal vacuoles (1-3) per cell, whereas class B mutants display abnormally fragmented vacuoles. In class C *vps* mutants there are no identifiable vacuoles, and class D mutants show a single, large vacuole that fails to extend into daughter buds. Finally, class E *vps* mutants show large vacuoles, with a very large and aberrant late endosome/MVB (the class E compartment) adjacent to the vacuole (Bowers & Stevens, 2005; Feyder *et al.*, 2015).

Proteins destined to the vacuole interact with different effectors such as the AP-1 complex (Phan *et al.*, 1994), epsins Ent3/Ent5 (Duncan *et al*, 2003), and the Golgi-associated gamma-adaptin ear homology domain Arf-binding proteins (GGAs) (Hirst et al., 2000; Pelham, 2000). These effectors ensure the formation of vesicles at the TGN by efficient recruitment of the clathrin coat. The TGN-derived vesicles fuse to endosomal membranes (Fig. 7). CORVET (core class C vacuole/endosome tethering) is the endosomal tethering complex, and contains the class C Vps11, Vps16, Vps18 and Vps33 proteins (class C core) and the class D Vps3 and Vps8 (Peplowska *et al.*, 2007). After being delivered to the endosomes, the cargo is directed towards the vacuole, by eventual fusion of the late endosome and the vacuole. This fusion requires the tethering complex HOPS (homotypic fusion and protein sorting), which is composed of the class C core and the class B Vps39 and Vps41 (Fig. 7). Additionally Vam3 (vacuole SNARE)

and the Ypt7 GTPase (homologous to the mammalian Rab7) are essential for the fusion of the late endosome to the vacuole (Peplowska *et al.*, 2007; Wickner, 2010).

Two types of proteins are destined to the vacuole via the VPS pathway: vacuolar membrane proteins (e.g., the vacuolar ATPase) that remain at the endosomal membrane and vacuolar soluble proteins (e.g., vacuolar protease) that are delivered to the endosomal lumen. The best example of a soluble protein targeted to the vacuole is the CPY, and for this reason the VPS pathway is also named as CPY pathway (Fig. 7). The Golgi-modified precursor form of CPY, named p2CPY, binds to Vps10 and the complex (p2CPY/Vps10) is sorted towards the late endosome, where it is cleaved to generate mature CPY (mCPY) (Bowers & Stevens, 2005). On the other hand, a third type of proteins, namely membrane-spanning proteins that have to reach the vacuolar lumen (e. g. carboxypeptidase S, Cps1), need to undergo an additional sorting step into vesicles budding into the lumen of the late endosome/MVB. Then the MVB content will be released into the vacuolar lumen by fusion of the MVB and the vacuole. All membrane proteins should be ubiquitinated by the Rsp5 ubiquitin ligase to be recognized by the sorting machinery at the MVB (Lauwers *et al.*, 2010). The machinery (MVB sorting machinery) that ensures the budding into the endosomal lumen is composed of class E Vps proteins, and consecutively assembled into four complexes named endosomal sorting complex required for transport (ESCRT) (Fig. 7), each having a specific function (table IV) (Bowers & Stevens, 2005; Henne *et al.*, 2011).

The ESCRT-mediated vesicles or intralumenal vesicles (ILV) formation and budding consist of five different stages, involving changes in the membrane and action of ESCRT complexes. All stages are summarized in figure 8 (Henne *et al.*, 2011).

Table IV. Proteins of the yeast ESCRT complex

Complex	Membrane binding	Cargo recognition	Intercomplex interactions
ESCRT-0 **-Hse1** **-Vps27**	PI(3)P via FYVE domain on Vps27	Via UIM (Ubiquitin-Interacting Motif) and VHS (**V**PS-27, **H**rs and **S**TAM) domains on Vps27 and Hse1	Vps27-Vps23 (ESCRT-I)
ESCRT-I **-Vps23** **-Vps28** **-Vps37** **-Mvb12**	Weak electrostatic interactions on Vps37	Via UEV (Ubiquitin E2 variant) domain on Vps23 and UBD (ubiquitin-binding domain) on Mvb12	Vps23-Vps27 (ESCRT-0) and Vps28-Vps36 (ESCRT-II)
ESCRT-II **-Vps22** **-Vps25** **-Vps36**	PI(3)P via GLUE domain of Vps36	Via GLUE (GRAM-Like Ubiquitin-binding in EAP45) domain of Vps36	Vps38-Vps28 (ESCRT-I) Vps25-Vps20 (ESCRT-III)
ESCRT-III **-Vps2** **-Vps20** **-Vps24** **-Snf7**	Myristoylation of Vps20	Interaction with DUBs (Deubiquitinating Enzymes) to deubiquinate cargo	Vps20-Vps25 (ESCRT-II) and MIM domain of 4 subunits of ESCRT III-Vps4
Vps4 complex **-Vps4** **-Vps60** **-Vta1**			MIM domain of ESCRT-III subunits via MIT domain

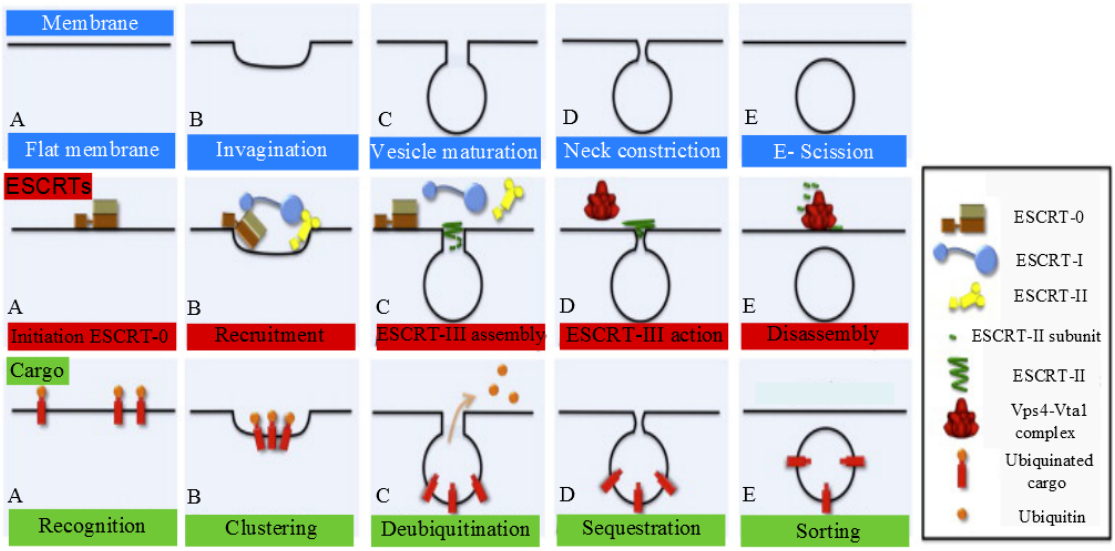

Figure 8. Formation of the ESCRT-mediated vesicle. The blue overview shows the events on the membrane. (A) The flat membrane (B) undergoes invagination (C) and complete maturation into a vesicle that is attached to the membrane by a neck that (D) undergoes constriction and leads (E) to the scission of the vesicles. The red and the green overviews are from the perspective of the ESCRT complex proteins and the cargo respectively, (A) ESCRT-0 always initiates the pathway by recognition of the ubiquitinated cargo, followed (B) by the recruitment of both ESCRT-I and ESCRT-II, leading to the clustering of the cargo (cargo enriched zone). (C) ESCRT-III is assembled, and the cargo is deubiquinated, (D) followed by the sequestration of the cargo into the vesicle due to the action of the ESCRT-III. (E) The ESCRT complex is disassembled by the Vps4-Vta1 complex, where finally the cargo is sorted upon vesicle budding (Henne *et al*., 2011).

III.1.3 Traffic from the Golgi to the vacuole via ALP pathway

Vesicles formed at the TGN can be directly sorted towards the vacuole via the ALP pathway (Fig. 7). This pathway was first described in a screen of factors involved in transport of alkaline phosphatase (ALP) from Golgi to vacuole. Deletion of adaptor protein-3 (AP-3) caused mis-localization of ALP but had no effect on the vacuolar transport of membrane-bound carboxypeptidase S (CPS) or soluble carboxypeptidase Y (CPY), indicating that AP-3 functions in the cargo-selective protein transport of ALP from Golgi to the vacuole (Cowles & Emr, 1997). In contrast, in vacuolar protein sorting (*vps*) mutants (deficient in the fusion of TGN-derived vesicles with the endosomes), ALP was correctly delivered to the vacuole, indicating the existence of a pathway independent of the endosomes that was named “ALP pathway”. The adaptor complex AP-3 (composed of subunits Ap16, Ap15, Apm3 and Aps3) forms the

machinery required for the ALP pathway (Odorizzi *et al.*, 1998a), which relies on the Ypt7 GTPase (homologous to the mammalian Rab7) and the HOPS tethering complex for the fusion of the TGN- derived vesicles with the vacuole (Wickner, 2010).

III.1.4 Endosome to Golgi retrieval pathway

Some proteins transported to endosomes are not delivered to the vacuole but transported from endosomes to the Golgi (Fig. 7). In yeast, the system responsible for this transport is named retromer complex. It was first identified in *S. cerevisiae*, and was found to recycle the CPY receptor Vps10 to the TGN. This complex is formed of five proteins that can be divided into two sub-complexes: a trimer of Vps26, Vps29, and Vps35, which are responsible for cargo selection, and a dimer of Vps5 and Vps17. These last two proteins belong to the family of sorting nexins (Snx), and mediate vesicle formation (Seaman *et al.*, 1997, Seaman *et al.*, 1998, Horazdovsky *et al.*, 1997).

IV. PHOSPHOINOSITIDES IN YEAST

IV.1 Phosphoinositides: Generalities

The term phosphoinositides refer to all phosphorylated cognates of phosphatidylinositol (PI). The inositol ring of PI can be reversibly phosphorylated at D3-, D4- or D5- positions. generating PI(3)P, PI(3,5)P_2, PI(3,4,5)P_3, PI(4)P, PI(4,5)P_2 and PI(5)P. In yeast, only PI(4)P, PI(4,5)P_2, PI(3)P, and PI(3,5)P_2 have so far been encountered (Divecha & Halstead, 2004; Strahl & Thorner, 2007) (Fig. 9). Different phosphoinositides have been shown to have a function as membrane signals, therefore contributing to specificity in membrane transport. This is supported by the fact that phosphatidylinositol (substrate), and phosphoinositides (products), as well as the kinases and phosphatases (modifying enzymes) are restrained to specific compartments in the cell (Strahl & Thorner, 2007).

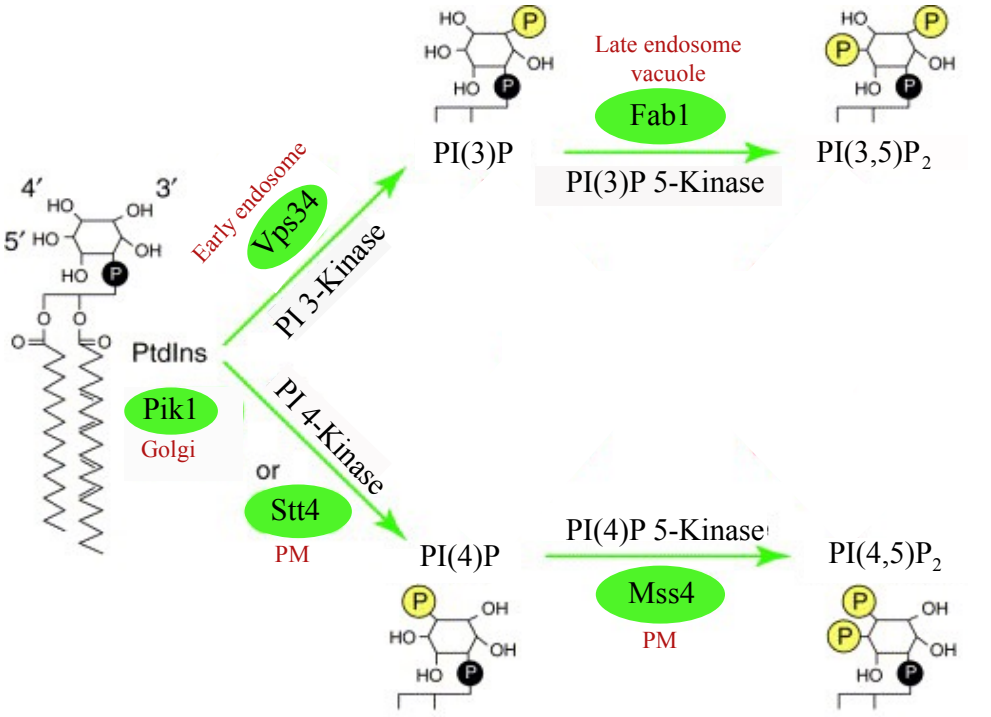

Figure 9. Pathways for the synthesis of phosphoinositides in yeast.

Schematic representation of the implicated phosphoinositide kinases, and their subcellular localization.

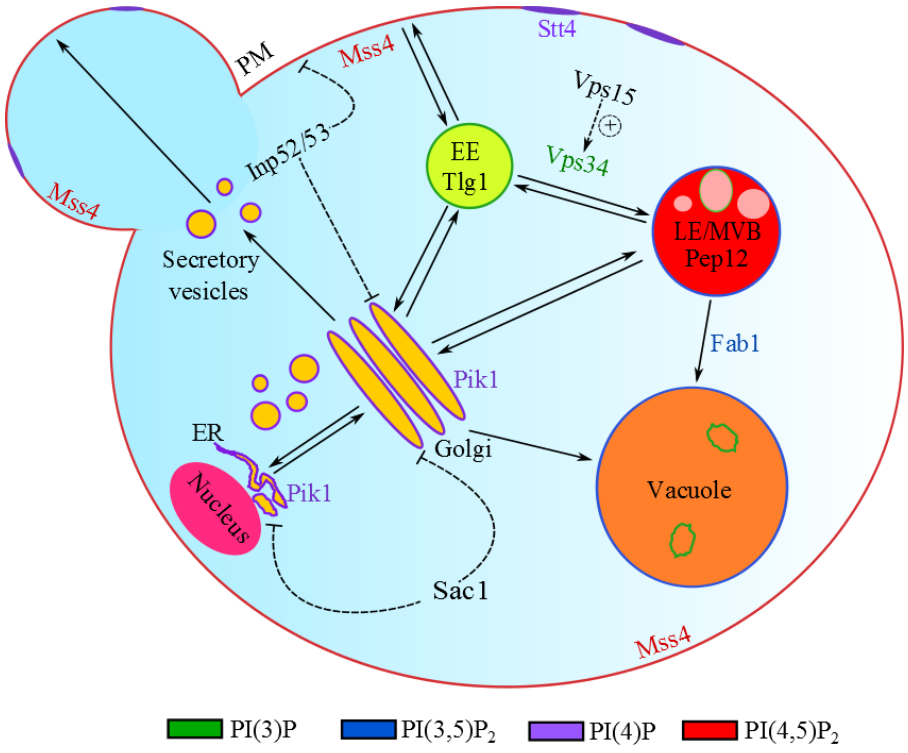

Figure 10. Distribution of phosphoinositides in yeast. PI(3)P is mainly synthesized in early endosomes by the PI 3-kinase Vps34. $PI(3,5)P_2$ is generated from the PI(3)P pool by the action of the PI(3)P 5-kinase Fab1 at the late endosome and the vacuole. PI(4)P can be generated from two main pools at the plasma membrane and the Golgi and nucleus, involving the phosphatidylinositol-4-kinases Stt4 and Pik1 respectively. $PI(4,5)P_2$ is synthesized in the plasma membrane by PI(4)P 5-kinase Mss4 from the conversion of its precursor PI(4)P.

IV.2 PI(3)P

Vps34 is the exclusive PI 3-kinase described in yeast (Marat & Haucke, 2016) and it was isolated in a screen for mutants defective in sorting from endosome to vacuole (reviewed in Marat & Haucke, 2016). Vps34 is present in two complexes in yeast, each having different location and function. Complex I contains Vps15, Vps30, Atg14 and Atg38, while complex II contains Vps15, Vps30, and Vps38. They are involved in autophagy and vacuolar protein sorting respectively (Strahl & Thorner, 2007; Funderburk *et al.*, 2010; Rostislavleva *et al.*, 2015; Marat & Haucke, 2016) In order to exert its activity, Vps34 has to be recruited to cellular membranes from the cytosol and activated by the Ser/Thr kinase Vps15 (Fig. 10). Despite the fact that Vps34 has been localized to both endosome and Golgi (Stack *et al.*, 1993; Stack, 1995; Rostislavleva *et al.*, 2015), it is considered that Vps34 alternatively acts at early

endosome compartments, and is not directly involved in the sorting from the Golgi (Gurunathan *et al*., 2002). A conserved RING domain called FYVE domain (whose name reflects the first four proteins found to contain it: Fab1p, YOTB, Vac1p and Early Endosome Antigen 1), is essential for recruitment of PI(3)P-binding proteins to membranes in both mammalians and yeast cells. In addition, PI(3)P can also bind to proteins displaying the Phox homology domain (PX) (Cullen, 2001). PI(3)P has been found to play a general role in endocytic traffic mediated by such FYVE and PX domains-containing effector proteins (Birkeland & Stenmark, 2004). Moreover, in specialized phagocytic cells PI(3)P is, involved in autophagy, as replacement of the wild type allele with a mutant in the lipid kinase domain block the autophagic pathway, and *vps34Δ* mutant yeast cells also show inhibition of autophagy (Kihara *et al*., 2001; Obara *et al*., 2008; Obara & Ohsumi, 2011).

IV.3 PI(3,5)P_2

PI(3,5)P_2 is one of the less abundant phosphoinositides in yeast (<0,01% of phospholipids), exerting its functions through binding to effector proteins, such as Ent3 and Ent5, in late endosome or vacuoles (Takatori *et al.*, 2016) (Fig. 10). Fab1 is a FYVE domain harboring protein, so it is among the PI(3)P effectors above described. Fab1 is the PI(3)P 5-kinase and generates PI(3,5)P_2 from the PI(3)P pool (Fig 9). Fab1 is not necessary for Golgi to vacuole trafficking; however *fab1*Δ mutant cells show an increased vacuole with reduced vacuolar hydrolases due to defects in vacuolar acidification (Gary *et al.*, 1998). In addition, Fab1 participates in the formation of the multivesicular body (MVB) and/or the fusion of endosomes with the vacuoles (Wurmser & Emr, 1998). Also Fab1 plays a role in the transport of cargoes from the vacuole to the Golgi, since *S. cerevisiae fab1*Δ mutants lacking detectable levels PI(3,5)P_2 show defects on such retrograde trafficking (Bryant *et al.*, 1998). In addition to that, Fab1 participates in the sorting of intra-luminal vacuolar proteins, since carboxypeptidase S is defectively sorted a in a *fab1*Δ mutant (Odorizzi *et al.*, 1998).

The Fab1 complex in *S. cerevisiae* consists of 3 main components: Fab1, Fig4, and Vac14 (Fig. 11B), which are conserved in mammalian cells (PIKfyve, Sac3 and Vac14 respectively). These components assure the synthesis and the turnover of PI(3,5)P_2. Fig4 is a PI(3,5)P_2 5-phosphatase that plays the role of negative regulator of

PI(3,5)P_2 signaling (Ho *et al.*, 2012). Vac14 acts as a scaffold protein that nucleates the assembly of the Fab1 complex (Jin *et al.*, 2008) and harbors HEAT motifs (helix-loop-helix) essential for protein-protein interactions (Fig. 11A) (Jin *et al.*, 2008; Dove *et al.*, 2009). Vac14 and Fig4 form a stable complex by binding directly to each other and are mutually dependent for interactions with Fab1, which recruits this complex to the vacuole (Rudge *et al.*, 2004; Botelho *et al.*, 2008).

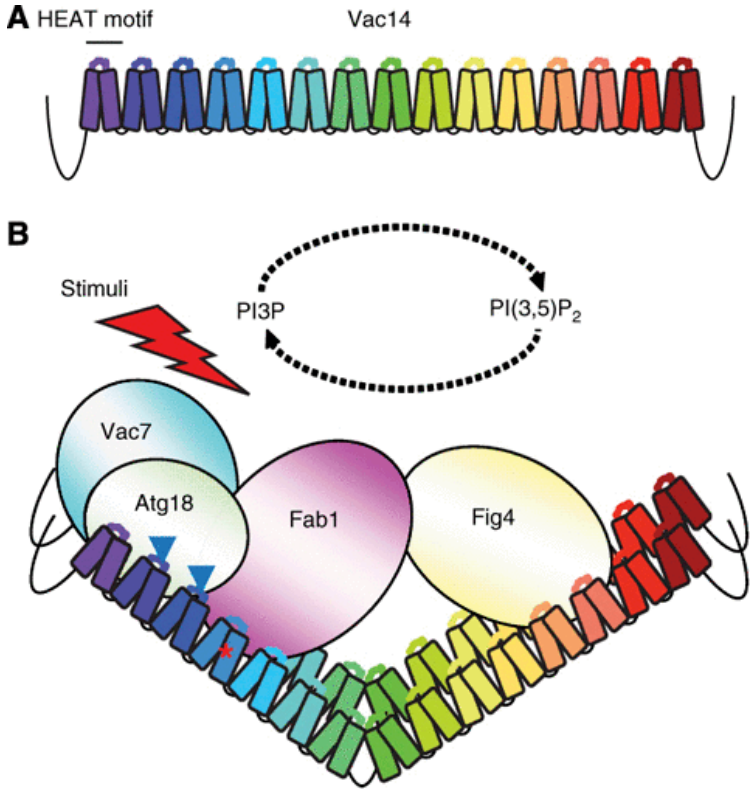

Figure 11. Fab1 complex. (**A**) Schematic presentation of Vac14. (**B**) Fig4 is a PI(3,5)P_2 5-phosphatase. Fig4 and Fab1 bind to distinct regions of Vac14. Vac14 binds to both Fab1 and Fig4 and may undergo a conformational change that brings Fab1 into contact with Fig4. Other components of the complex that also interact with Vac14 areVac7 and Atg18 (Jin *et al.*, 2008).

IV.4 PI(4)P

In yeast there are three PI 4-kinase encoding genes, *STT4*, *LSB6*, and *PIK1*. Lsb6 is not essential for yeast viability, and its contribution to cellular PI(4)P levels seems to be minor, whereas Stt4 and Pik1 are both essential and responsible for the bulk of this phosphoinositide (Yoshida *et al.*, 1994a; Han *et al.*, 2002). However, the overexpression of Lsb6 partially suppresses the lethal phenotype of an *stt4* mutant and localizes at the

plasma membrane and vacuolar membrane in yeast (Han *et al*., 2002). Thus, most PI(4)P in yeast can be generated from two spatially distinct pools. The first one is at the PM level, and involves the phosphatidylinositol-4-kinase Stt4. And the second one involves Pik1, that generates PI(4)P at the Golgi and the nucleus (Garcia-Bustos *et al.*, 1994; Cutler, 1997; Strahl *et al*., 2005). Due to the different localization of both kinases, the two pools of PI(4)P remain functionally separated. The reservoir of PI(4)P at the Golgi has an important role in the secretory route (formation of secretory vesicles), as evidenced by the fact that a *pik1* mutant shows defects in Golgi-plasma membrane traffic (Hama *et al*, 1999). PI(4)P is an essential regulator of Golgi function, such as that of the TGN sorting hub, and it is involved in exocytosis by regulation of the sequential activation of Rab family GTPases. Sec2 (RAB GEF) binds simultaneously to PI(4)P at the Golgi and to the Rab GTPase Ypt32. Then Sec2 also activates the Rab GTPase Sec4, which recruits the exocyst effector Sec15 (Odorizzi *et al*.,2000; Santiago-Tirado & Bretscher, 2011; Mayinger, 2012). However, high PI(4)P levels may inhibit docking or fusion of secretory vesicles to the plasma membrane (Ling *et al*., 2014). Also, in thermosensitive *pik1-101* mutant cells, the uptake of the endocytic marker FM4-64 is blocked at endosomes. In addition, Golgi-localized Pik1 is required for autophagy (Wang *et al*., 2012). Moreover, in a *pik1* mutant the anterograde transport form ER to Golgi was blocked indicating the necessity of PI(4)P in the fusion of COPII vesicles with the Golgi (Lorente-Rodríguez & Barlowe, 2011). In sum, PI(4)P pools at the Golgi are a key to multiple processes: in the SEC and END pathways, as well as in autophagy.

Stt4 is recruited to the PM through interaction with both Erf3 and Ypp1adaptors (Wu *et al*., 2014). Stt4 has been implicated in actin cytoskeleton organization and vacuolar morphology. This protein is required for normal trafficking of the essential aminophospholipid phosphatidylserine from the endoplasmic reticulum (ER) to the Golgi/vacuole, but its activity is dispensable for protein trafficking between these compartments (Audhya *et al*., 2000). In addition, PI(4)P at the plasma membrane has an important role in cell signaling and maintenance of cell wall integrity in yeast. The first evidence of its participation in signaling was based on the fact that the phenotype of sensitivity to staurosporine of a *stt4* mutant (Staurosporine and Temperature Sensitive) was rescued by overexpression of Pkc1 (Protein serine/threonine kinase of the yeast cell wall integrity MAPK pathway) (Satoshi *et al.*, 1994a). Moreover, PM-localized Stt4 has

been shown to be necessary for activation of the Pkc1-mediated Slt2 MAPK cascade (Audhya & Emr., 2002). It is also required for autophagy, as shown by the defect in trafficking to the vacuole of the ubiquitin-like protein Atg8 displayed by a *stt4-4* ts mutant (Wang *et al*., 2012). Furthermore, PI(4)P at the plasma membrane acts as precursor for the production of the essential pools of $PI(4,5)P_2$ by Mss4 (Roy & Levine, 2004).

IV.5 $PI(4,5)P_2$ a key to plasma membrane dynamic

Although $PI(4,5)P_2$ counts for only 1% of membrane phospholipids, it is the most abundant phosphoinositide in the yeast cell. In *S. cerevisiae* $PI(4,5)P_2$ is synthesized in the plasma membrane by the PI(4)P 5-kinase Mss4 (Multicopy Suppressor of Stt4) through the conversion of its precursor, the PI(4)P generated in the plasma membrane by Stt4 (Desrivieres *et al*., 1998). The *MSS4* gene is essential for yeast and was isolated as a suppressor of a thermosensitive *stt4* mutant (Yoshida *et al*., 1994b). Mss4 is implicated in the organization of the actin cytoskeleton during polarized growth (Desrivieres *et al*., 1998). $PI(4,5)P_2$ functions mainly at the plasma membrane level, by recruiting a diversity of proteins bearing specific $PI(4,5)P_2$-binding domains, involved in endocytosis, cell polarity or signal transduction (Sun *et al*., 2005; He *et al*., 2007; Fernández-Acero *et al*., 2015). In fact, it has been described that this phosphoinositide forms part of microdomains at the PM, specialized lipid rafts which act as signaling platforms by concentrating substrates and enzymes in the same environment (McLaughlin & Murray, 2005; Johnson & Rodgers, 2008). It has also been described the presence of $PI(4,5)P_2$ in the nucleus, where it seems to regulate RNA processing (Barlow *et al*., 2010; Keune *et al*., 2011), as well as in vacuolar membranes, where it cooperates with PI(3)P in coordinating ESCRT assembly for dynamic events (Mima & Wickner, 2009). Finally, this phosphoinositide has also been related to the regulation of transmembrane proteins, playing a role as cofactor of some ion channels and transporters (Suh & Hille, 2008).

IV.6 Regulation of phosphoinositides in yeast

In yeast, phosphoinositide levels are down-regulated by seven poly-phosphoinositide phosphatases that remove different phosphates (Fig. 12), which are grouped into three classes according to their catalytic domains (Strahl & Thorner, 2007, Hughes, 2000): proteins of the first class display a catalytic domain similar to the one in the Sac1 phosphatase (Sac1, Fig4, Inp51/Sjl1, Inp52/Sjl2 and Inp53/Sjl3); the second class encompasses proteins with a catalytic domain with similarity to PI 5-phosphatases that dephosphorylate phosphorylated substrates in the 5' position of the inositol ring (Inp51/Sjl1, Inp52/Sjl2, Inp53/Sjl3, and Inp54), which in addition to having a domain of this type also contain a Sac1 domain; the third group is represented by the Ymr1 phosphatase, which has similarity to mammalian myotubularin. As we can see in figure 12, many of these proteins display a redundant activity in yeast, although it seems that Sac1 acts preferably on PI(4)P (Foti *et al.*, 2001), Fig4 on $PI(3,5)P_2$ and the PI 5-phosphatases Inp51/Sjl1 and Inp54 on $PI(4,5)P_2$ (Strahl & Thorner, 2007). Regarding the functional involvement of these proteins, none of them is essential but their lack has been linked to defects in organization of the actin cytoskeleton, secretion, endocytosis, vacuolar function and cell wall maintenance (Strahl & Thorner, 2007).

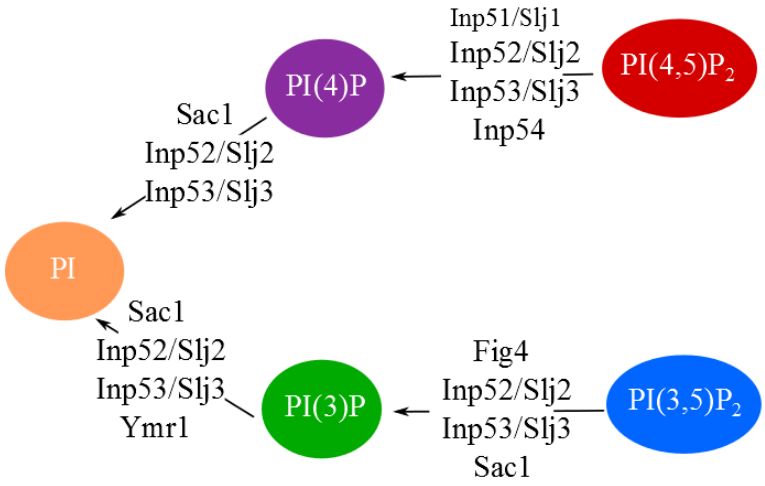

Figure 12. Substrate specificity of phosphoinositide phosphatases in yeast.

MATERIALS & METHODS

I. MICROORGANISMS

I.1 *Saccharomyces cerevisiae* strains

The yeast strains used all along this study are detailed in table V

Table V. *S. cerevisiae* strains

Strain	Genotype	Source
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
YPH499	*MATa ade2-10 trp1-63 leu2-1 ura3-52 his3-Δ20 lys2-801*	(Sirkoski & Hieter, 1989)
YES95	*YPH499 ; pik1-63::TRP1*	Roelants & Thorner (University of California at Berleley, CA, USA
YFR201	*YPH499 ; stt4Δ::HIS3* [CEN LEU2 *stt4-4ts*]	Roelants & Thorner (University of California at Berleley, CA, USA
SD18-1d	*MATa his3Δ leu2 rme1 ura3 trp1 mss4::HIS3MX6* [YCplac111-MSS4]	Desrivieres & Hall (Biozentrum University of Basel, Switzerland)
SD19-3a 1d	*MATa his3Δ leu2 rme1 ura3 trp1 mss4::HIS3MX6 [YCplac111-mss4-2ts]*	Desrivieres & Hall (Biozentrum University of Basel, Switzerland)
VHY87	*MATα leu2-3, 112 ura3-52 his4 can1^{R} TRP1::DsRed-HDEL*	M. Cyert, (Stanford University, CA, USA)
SEY6210	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 lys2-801*	Botelho et al (2008)
RBY64	*SEY6210; VAC14-HA::TRP1*	Botelho et al (2008)
Y00575	*BY4741; ypt7::kanMX4*	EUROSCARF
Y05072	*BY4741; vps10::kanMX4*	EUROSCARF
Y02783	*BY4741; vps16::kanMX4*	EUROSCARF
Y04329	*BY4741; vps3::kanMX4*	EUROSCARF
Y00405	*BY4741; vps8::kanMX4*	EUROSCARF
Y04462	*BY4741; vps45::kanMX4*	EUROSCARF
Y05381	*BY4741; vps27::kanMX4*	EUROSCARF
Y03416	*BY4741; vps23::kanMX4*	EUROSCARF
Y02826	*BY4741; vps22::kanMX4*	EUROSCARF
Y06211	*BY4741; vps20::kanMX4*	EUROSCARF
Y06211	*BY4741; vps10::kanMX4*	EUROSCARF
Y05072	*BY4741; vps2::kanMX4*	EUROSCARF
Y01370	*BY4741; vps26::kanMX4*	EUROSCARF
Y05295	*BY4741; vac14::kanMX4*	EUROSCARF

Table V. Continued

Strain	Genotype	Source
Y07080	*BY4741; fab1::kanMX4*	EUROSCARF
Y01120	*BY4741; fig4::kanMX4*	EUROSCARF
Y05700	*BY4741; atg18::kanMX4*	EUROSCARF
Y06152	*BY4741; bem2::kanMX4*	EUROSCARF
Y01146	*BY4741; cla4::kanMX4*	EUROSCARF
Y01370	*BY4741; pep8::kanMX4*	EUROSCARF
Y00975	*BY4741; vps29::kanMX4*	EUROSCARF
Y02132	*BY4741; vps30::kanMX4*	EUROSCARF
Y01271	*BY4741; vps35::kanMX4*	EUROSCARF
Y05269	*BY4741; vps38::kanMX4*	EUROSCARF
Y01933	*BY4741; ypt35::kanMX4*	EUROSCARF
Y04735	*BY4741; vma21::kanMX4*	EUROSCARF
Y06992	*BY4741; voa1::kanMX4*	EUROSCARF
Y00887	*BY4741; dyn3::kanMX4*	EUROSCARF
Y03883	*BY4741; vma1::kanMX4*	EUROSCARF
Y01323	*BY4741; lsb6::kanMX4*	EUROSCARF

I.2 Bacterial Strains

Escherichia coli DH5α (F– Φ80*lac*ZΔM15 Δ (*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1) was used routinely for plasmid amplification.

II. CULTURE MEDIA

The composition of the culture media used in this work is detailed in table VI. The water used for dissolution was deionized through Millipore purification systems. The media were autoclaved at 121°C and 1 atmosphere ovepressure for 20 minutes. To obtain solid media, 2% w/v agar were added to the usual composition.

Table VI. Culture media used for *E. coli*, and *S. cerevisiae*

Media	Composition	Utility	Source
LB (Luria-Bertani)	1% bactrotryptone, 0.5% yeast extract, and 0.5% NaCl	Rich medium that is commonly used in bacterial culture. Often ampicillin (100µg/ml) or kanamycin (50 µg/ml) are added to the sterilized medium to select for cells that contain a specific genetic element such as a plasmid resistant to one of these antibiotics	(Sambrook *et al.*, 1989)
YPD (Yeast extract Peptone Dextrose)	1% yeast extract, 2% peptone, and 2% glucose	Complete medium used for yeast growth	(Sherman *et al.*, 1986)
YPDA(Yeast extract Peptone Dextrose Adenine)	1% yeast extract, 2% peptone, 2% glucose, and 0.01% adenine	Rich medium supplemented with adenine to facilitate strains with *ade2* genotype	(Sherman *et al.*, 1986)
Synthetic medium SD: Synthetic Dextrose	2% glucose, 0.1% nitrogen base for yeast, 0.5% ammonium sulfate, and 0.19% of amino acids mix (Formedium)	Syntethic medium for yeast growth supplemented with correspondent amino acids necessary for the selection and maintenance of transformants harboring different auxotrophic markers.	(Sherman *et al.*, 1986)
Synthetic medium SR: Synthetic Raffinose	1,5% glucose, 0,1% nitrogen base for yeast, 0.5% ammonium sulfate, and 0.19% of amino acids mix (Formedium)	Same composition as SD medium, except for glucose that is replaced by raffinose to prevent the repression of the *GAL1* promoter	(Sherman *et al.*, 1986)

III. MANIPULATION OF MICROORGANISMS

III.1 General conditions of growth

The conditions of culture incubation were as following:

- *E. coli*: 37℃, and 200-220 rpm for incubations in liquid medium
- *S. cerevisiae*: 30℃ for normal growth conditions with agitation between 180 and 220 rpm. In the majority of experiments, samples were inoculated in liquid YPD or SD (when the strain used was transformed with a plasmid) and grown O/N until the culture reached the stationary phase of growth. Strains transformed with plasmids under the control of *GAL1* promoter were inoculated in SR medium O/N. The following day cells were inoculated at OD_{600} of 0.2 in SR liquid medium containing galactose 2% (w/v) and were incubated for 4-5 hours. The presence of galactose in culture medium induces the expression of genes under the control of *GAL1* promoter.

III.2 Strains conservation

Yeast strains were conserved at -80℃ in 50% (v/v) glycerol solution. The *E.coli* strains harboring plasmids were conserved as well at -80℃ in 25% (v/v) glycerol after their growth in LB liquid medium with the appropriate antibiotic.

III.3 Serial dilution drop growth assays

Starting from cultures in stationary phase where the density of cells was adjusted to an OD_{600} of 0.5, and serial decimal dilutions were realized in 96-well plates. Finally, with the help of the replicator (VP407AH Multi-blot replicator from V&P Scientific, Inc.) 5 µL of each dilution were seeded onto the appropriate solid medium. As the OD_{600} of 1 is approximately equal to 10^7 cells/mL, in the first drop $2.5x10^4$ cells were seeded, and 25 cells in the fourth and last drop. Plates were incubated for 48-72 hours at 30℃ and/or 37℃.

IV. MOLECULAR BIOLOGY TOOLS AND DNA MANIPULATION

For basic molecular biology techniques, like polymerase chain reaction (PCR), plasmid extraction from *E. coli* through alkaline lysis, restriction enzymes digestions, DNA 5´ end dephosphorylation, etc., standard protocols were used (Sambrook *et al*., 1989).

IV.1 Plasmid extraction from *E.coli*

Plasmids from *E.coli* were extracted using the alkaline lysis protocol and occasionally by using the commercial kit NZYMiniprep (NZYTECH). For the alkaline lysis protocol, *E.coli* was grown in LB medium with 100 µg/ml ampicillin at 37 °C overnight (O/N) with shaking. The following day 1.5 mL of the culture were transferred to an Eppendorf tube, and span down at high speed for 1 min at room temperature. The supernatant was discarded and 100 µl of resuspension solution (Buffer I: 50mM Glucose, 10mM EDTA, and 25mM Tris-HCl pH 8) were added to the tube, and mixed by vigorous vortexing until the pellet was completely re-suspended. Then 200 µl of freshly prepared lysis buffer (buffer II: 0.2 N NaOH, 1% SDS) were added, and mixed gently by inverting the tube 6-8 times until the solution turned transparent and became more viscous. Finally the neutralizing buffer was added (Buffer III: 29.4% potassium acetate and 11.5% of acetic acid glacial) and mixed my inverting the tube 6-8 times. The mix was centrifuged at 13,000 rpm for 5 min and the supernatant was precipitated by 2 volumes 100% ethanol at -20℃ for 20 min, followed by 10 min centrifugation and washing with 70% ethanol. The pellet was dried by leaving the tube open at 37℃ in a heat blocker until the ethanol was evaporated and the pellet was re-suspended in 20µL of distilled DNAse-free water. When DNA extracted was used for sequencing, the NZYMiniprep kit was used following the manufacturer's instructions.

IV.2 Transformation of *E.coli*

For plasmid transformation, the protocol developed by Hanahan (1983) was used as follows: 100 µL of competent cells were incubated for 30 min on ice with 1 µL of DNA plasmid or with 10 µL of DNA from the ligation reaction. Subsequently a thermic shock was performed for 90 sec at 42℃ and cells were again incubated 2 min

on ice. Then 4 volumes of LB were added to the mix and cells were incubated for 1 h shaking at 37℃. Finally, cells were span down and the pellet was cultured in LB plates with 100 µg/ml ampicillin at 37℃ for 24 h.

IV.3 Yeast transformation

In order to transform yeast, the conventional transformation protocol with lithium acetate (Ito *et al*., 1983) was used as follows: from a pre-inoculum in YPD, yeast cells were grown until they reached an OD_{600} of 1. Ten mL of the culture were taken and re-suspended with a mix of 40% of polyethtylene glycol 4000, 0.2M lithium acetate and 1µL/mL dithiothreitol (DTT). 0.6 mg of DNA were added and incubated for 10 min at room temperature. Subsequently cells were incubated for 30 min at 45℃, and then inoculated in selective media SD, in function of the auxotrophic marker of the plasmid for 3 days at 30℃.

IV.4 DNA manipulations and cloning

The DNA oligonucleotides (Sigma) and plasmids used in this work are detailed in tables VII and VIII respectively. To construct plasmids pYES2-GFP-SteA^{WT} and pYES3-GFP-SteA^{WT} (Table VIII), we used both pEG(KG)-SteA^{WT} and pYES3-GFP. First, plasmid pEG(KG)-SteA^{WT} was digested by *Bam*HI and the SteA-encoding DNA fragment was ligated into that site of pYES2-GFP or pYES3-GFP plasmids. To construct pYES3-GFP-derivatives expressing mutant SteA proteins (GFP-SteA^{K29A}, GFP-SteA^{K33A}, GFP-SteA^{K36A}, GFP-SteA^{3K-3A}, and GFP-SteA$^{2k2R-4A}$, DNA of pGEX-4T-2 derivatives bearing *steA* mutant alleles (provided by L. J. Mota, ITBQ, Lisbon) was digested with *Bam*HI and *Xho*I and the appropriate DNA fragments were ligated into equivalent sites of pYES3-GFP. The accuracy of the nucleotide sequence of the inserts in the constructed plasmids was checked by DNA sequencing at the Centro de Genómica UCM. To construct plasmid pEG(KG)-SteA^{K36A}, an overlapping PCR was performed using the pEG(KG)-SteA^{WT} as template and 4 oligonucleotides, 2 external to amplify the whole gene (SteA-1 and SteA-2) and 2 internal harboring the point mutation (K36A-F and K36A-R) (Table VII). Then the PCR product was digested with *Bam*H1 and cloned into pEG(KG).

Enzymes and kits used for plasmid constructions were Expand high fidelity PCR system (Roche), restriction enzymes (NZYTEC, and Sigma), T4 DNA Ligase (Life Technologies and Roche), DNA clean & concentrator GeneClean® turbo kit (MPbio), according to the instructions of the manufacturers.

IV.5 Oligonucleotides used

The oligonucleotides (Sigma) used in this study are detailed in the following table VII (the sequences recognized by the restriction enzymes are underlined).

Table VII. Oligonucleotides used in this study and their usage**.** The sequences recognized by the restriction enzymes are underlined.

Oligonucleotide	Sequence	Utility
SteA-1	CGGGATCCATGCCATATACATCAGTTTCT	Used to construct pEG(KG)-SteA
SteA-2	CGGGATCCTTAATAATTGTCCAAATAGTTATG	Used to construct pEG(KG)-SteA
SteA2-GFP	CGGGATCCATAATTGTCCAAATAGTTATGG	Used to construct YCpLG-SteA-GFP
SteA2-GFP	CGGGATCCATAATTGTCCAAATAGTTATGG	Used to construct pYES3-GFP-SteA
K36A-F	ATAAATTATCAACAAAAATCATGGCAGGCATCTTGTATGTGCTTACCGCAG	Used to construct pEG(KG)SteA^{K36A}
K36A-R	CTGCGGGTAAGCACATACAAGATGCCTGCCATGATTTTTGTTGATAATTTAT	Used to construct pEG(KG)-SteA^{K36A}

Table VIII. Plasmids used in this work and their characteristics

Name	Characteristics and use	Source
pGEX-SteA^{3K-3A}	Derivative of pGEX-4T-2 encoding GST-SteA with lysines 29, 33 and 36 of SteA substituted by alanines (AmpR)	(Dominguez *et al.*,2015)
pGEX-SteA2K2R-4A	Derivative of pGEX-4T-2 encoding GST-SteA with lysines 154 and 156, and arginines 159 and 161 of SteA substituted by alanines (AmpR)	(Dominguez *et al.*,2015)
pGEX-SteA^{K29A}	Derivative of pGEX-4T-2 encoding GST-SteA with lysine 29 of SteA substituted by alanine (AmpR)	(Dominguez *et al.*,2015)
pGEX-SteA^{K33A}	Derivative of pGEX-4T-2 encoding GST-SteA with lysine 33 of SteA substituted by alanine (AmpR)	(Dominguez *et al.*,2015)
pGEX-SteA^{K36A}	Derivative of pGEX-4T-2 encoding GST-SteA with lysine 36 of SteA substituted by alanine (AmpR)	(Dominguez *et al.*,2015)
pEG(KG)	Vector to express GST fusion proteins in yeast (*URA3*)	(Mitchell *et al.*, 1993)
pEG(KG)-SteA	Derivative of pEG(KG) encoding GST-SteA (*URA3*)	Isabel Rodríguez-Escudero
pEG(KG)-SteA^{K36A}	Derivative of pEG(KG) encoding GST-SteA with lysine 36 of SteA substituted by alanine (*URA3*)	This study
pEG(KG)-SteA$^{(98-210)}$	Derivative of pEG(KG) encoding GST-SteA carboxy terminus (*URA3*)	Isabel Rodríguez-Escudero
YCpLG-GFP	Yeast galactose-inducible expression vector (*LEU2*)	(Rodríguez-Escudero *et al.*, 2005)
YCpLG-SteA^{WT}-GFP	Derivative of YCpLG encoding SteA^{WT} fused to the N-terminus of GFP (*LEU2*)	Isabel Rodríguez-Escudero
YCpLG-SteA$^{(1-97)}$-GFP	Derivative of YCpLG encoding SteA amino terminus fused to the N-terminus of GFP (*LEU2*)	Isabel Rodríguez-Escudero
pYES2	Vector for inducible expression of recombinant proteins in *S. cerevisiae* (*URA3*)	Life Technologies
pYES2-GFP	Derivative of pYES2 encoding GFP (*URA3*)	(Rodríguez-Escudero *et al.*, 2005)
pYES2-GFP-SteA^{WT}	Derivative of pYES2-GFP encoding SteAWT fused to the C-terminus of GFP (GFP-SteAWT) (*URA3*)	This study
pYES3/CT	*S. cerevisiae* expression vectors derived from the parental pYES2 vector (*TRP1*)	Life Technologies
pYES3-GFP	Derivative of pYES3/CT encoding GFP	(Rodríguez-Escudero *et al.*, 2005)
pYES3-GFP-SteA^{WT}	Derivative of pYES3-GFP encoding SteAWT-GFP (*TRP1*)	This study

Table VIII. Continued

Name	Characteristics and use	Source
pYES3-GFP-SteA^{K29A}	Derivative of pYES3-GFP encoding SteA-GFP with lysine 29 of SteA substituted by alanine (*TRP1*)	This study
pYES3-GFP-SteA^{K33A}	Derivative of pYES3-GFP encoding SteA-GFP with lysine 33 of SteA substituted by alanine (*TRP1*)	This study
pYES3-GFP-SteA^{K36A}	Derivative of pYES3-GFP encoding SteA-GFP with lysine 36 of SteA substituted by alanine (*TRP1*)	This study
pYES3-GFP-SteA^{3K-3A}	Derivative of pYES3-GFP encoding SteA-GFP with lysines 29, 33 and 36 of SteA substituted by alanines fused to C-terminal of GFP (*TRP1*)	This study
pYES3-GFP-SteA$^{2K2R-4A}$	Derivative of pYES3-GFP encoding SteA-GFP with lysines 154 and 156, and arginines 159 and 161 substituted by alanines (*TRP1*)	This study
YCpLG-PI3K	Derivative of YCpLG encoding the catalytic subunit (p110α) of mammalian class I phosphatidylinositol 3-kinase	(Rodríguez-Escudero *et al*., 2005)
YCpLG-PI3K(K802R)	Derivative of YCpLG-PI3K bearing a catalytically inactive ('kinase-dead') allele of mammalian class I PI3K.	(Rodríguez-Escudero *et al*., 2005)
pEG(KG)-SteA$^{(1-97)}$	Derivative of pEG(KG) encoding GST-SteA amino terminus (*URA3*)	Isabel Rodríguez-Escudero
pRS426GFP2xPH(PLCδ)	Plasmid constitutively expressing a fusion of GFP to two copies in tandem of the PH-domain of PLCδ (*URA3*)	(Stefan *et al*., 2002)
YEPlac112-ILV6-mCherry	Derivative of YEplac112 expressing the mitonchondrial marker ILV6-mCherry (*TRP1*)	(Rodríguez-Escudero *et al*., 2011)
pGPD426-Lact-C2mCherry	Yeast expression plasmid encoding Lact-C2-mCherry	(Fairn *et al*., 2011)
pEG(KG)-SopB^{WT}	Derivative of pEG(KG) encoding GST-SopB (*URA3*)	(Rodríguez-Escudero *et al*., 2005)
pEG(KG)-SopBR^{468A}	Derivative of pEG(KG) encoding GST-SopB with arginine 468 of SopB substituted by alanine (*URA3*)	(Rodríguez-Escudero *et al*., 2005)
pMR072	HA-VOA1 (1xHA inserved after Asp25. Plasmid is pRS316-based	(Ryan *et al*, 2008)
pESC-TRP-mCherry-SidC	Derivative of pESC-TRP	Julia M. Coronas-Serna

V. PROTEIN DETECTION THROUGH WESTERN-BLOTTING

V.1 Preparation of yeast protein extracts

The yeast samples for protein immunodetection were collected on ice in cold Falcon tubes (10 ml for cells suspensions at OD_{600}=1), centrifuged to eliminate any remaining media and re-suspended in 100µl of ice-cold lysis buffer (50 mM TrisHCl pH=8, 10% [v/v] glycerol, 1% [v/v] TritonX-100, 0.1% [w/v] SDS, 150 mM NaCl, 50 mM NaF, 1 mM sodium orthovanadate, 50 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 5 mM EDTA pH=8 and protease inhibitors) at 4 ℃ in Eppendorf tubes. The protein extracts were obtained through mechanical rupture by glass beads (Sartorius) as described (Martin *et al.*, 2000), adapting the rupture pulses in the Fast-prep (FP120/BIO101 ThermoSavant) to 3 repeats of 30 seconds. The protein concentration of the supernatants was measured at 280 nm in a spectrophotometer Beckman DU 640 and normalized with loading buffer 2x (Tris-HCl 0.1 M pH=6.8, 20% [v/v] glycerol, 4% [w/v] SDS, bromophenol blue and 10% DTT 1M). The extracts were finally boiled for 10 min. and centrifuged to eliminate potential cellular debris prior to their load into the polyacrylamide gel.

V.2 Proteins electrophoresis

For the separation of the proteins from the extract, polyacrylamide gels constituted by a 5% concentrator gel and an 8%, 10% or 15% separator gel under denaturing conditions with SDS were used. It was done in cuvettes (Bio-Rad) at a constant voltage of 150-180 V. An electrophoresis buffer was used with the following composition: 196mM glycine, 0.1% SDS, 50mM Tris-HCl pH 8.3. The molecular weight marker used was provided by Invitrogen or Bio-Rad.

Once proteins were separated on the polyacrylamide gel, they were transferred from the gel to nitrocellulose membranes (Hybond, Amersham) and paper Whatman 3MM Chr was used as protective and absorbent material. Transfer cuvettes were used (Bio-Rad) at a constant voltage of 100 V for 1 h. The transfer buffer used is composed of 5.8 g/l Tris, 2.9 g/l glycine and 0.37 g/l SDS, and 20% of the final volume of ethanol was added just prior to use.

V.3 Immunodetection

Membranes were probed with the appropriate antibodies and developed according to the manufacturer´s conditions using the Quantitative Fluorescent Imaging System Odyssey from Li-Cor Biosciences. Membranes were blocked for at least 1 h at room temperature (or overnight at 4℃) with 5% skimmed milk. TPBS buffer (PBS plus Tween-20) was used for incubations and washes. After blocking, membranes were incubated with the primary antibody in 1% skimmed milk for 2h at RT or overnight at 4℃. Then the membranes were washed several times with TPBS and incubated with the secondary antibody, also in 1% skimmed milk (≈1h at RT) in the dark. The primary antibodies used were, rabbit polyclonal anti-G6PDH (Sigma) at 1:50.000, rabbit polyclonal anti-GST (Santa Cruz Biotechnology) at 1:200, rat monoclonal anti-HA clon11 (Covance) at 1:1000, rat monoclonal anti-GFP (JL-8) (Clontech) at 1:2000, and rat monoclonal anti-HA high affinity (100 ng/ml) (Roche).

The secondary antibodies used were: IRDye 800CW goat anti-rabbit (Green), IRDye 680CW Goat anti-rabbit (Red) and IRDye 680CW goat anti-rabbit (Red) all at 1:5000 (Li-Cor Biosciences).

VI. YEAST LIVE-CELL IMAGING

Microscopy of live yeast cells for GFP detection was performed in an Eclipse TE2000U microscope (Nikon, Melville, NY, USA) fitted with an Orca C4742-95-12ER digital camera (Hamamatsu, Japan) and then processed by using HCImage System (Hamamatsu, Japan). Yeast cells harboring GFP fusion vectors were cultured overnight in selective SR medium and then refreshed in the same medium to adjust OD_{600} to 0.2 at the time of adding galactose to a 2% (w/v) concentration. After 4 h at 30ºC, cells suspensions were mounted on slides for live microscopy observation. For *ts* mutant strains, cells were refreshed and incubated in the presence of 2% (w/v) galactose at 24ºC for 4 h and then shifted to 37ºC for 2 h. For FM4-64 (Molecular Probes TM, ThermoFischer Scientific) staining, the fluorochrome was added to a cell suspension in SR medium to a concentration of 24 µM and incubated for 60 min prior to observation. In order, to stain the vacuolar lumen, the marker 7-amino-4-chloromethylcoumarin (CMAC, C2110 Molecular Probes, Invitrogen) was added to a final 10 µM

concentration in 100 µL of culture and incubated for 5 minutes at 25 ℃ Then, the cells were washed three times with PBS before microscopic observation.

VII. CO-PURIFICATION ASSAY (GST Pull-down)

Transformants were grown overnight in selective SR medium. The next day, cells were sub-cultured in SR medium supplemented with galactose with final concentration of 2%, for 6 h, thus inducing protein expression in fusion to different epitopes. Protein extracts were obtained as described in section V.1 but varying slightly the composition of lysis buffer, which in this case contained less detergent (without SDS or Triton X-100) in order to promote co-purification of proteins. After equaling the amount of protein, a small sample was taken to be used as raw extract control, which was processed by boiling in loading buffer. On the remaining sample 30 µL of the matrix Glutathione Sepharose 4B (GE Healthcare Life Sciences) were added, previously equilibrated with 50% lysis buffer. In order to foster contact with the matrix, extract volume was increased (to 300 µL) with lysis buffer. After one hour incubation at 4 °C with stirring rotation, three washings of the matrix were performed with the same cold buffer and finally samples were eluted by boiling the sample in 20 µL of loading buffer 2X SDS-PAGE for 5 mins in order to separate the proteins from the matrix. These samples were loaded onto polyacrylamide gels for immunodetection by Western blotting, along with the raw extracts previously separated.

VIII. *IN VIVO* CO-IMMUNOPRECIPITATION OF MEMBRANE PROTEINS USING CROSSLINKER DSP

Cells transformed with different combinations of plasmids were grown overnight in SR medium. The next day cells were sub-cultured in 100 mL of SR medium supplemented with galactose 2% (v/v) until the cultures reached an OD_{600} of 0.5-0.7. Afterwards cells were harvested and re-suspended in 5 mL PBS, and the crosslinker DSP (Thermo Scientific) was added at a final concentration of 2.5m M for 15 mins in agitation. Then 500 µL of Tris-HCl pH 8 20mM were added for 15 mins in order to stop the reaction. Cells were harvested again by centrifugation at 3000 rpm for 3 mins.

For protein extraction, treated cells were washed once with MilliQ water, re-suspended in 500 µl of RIPA buffer [50 mM Tris pH 7.6, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% deoxycholic acid] and vortexed 3 times along with 0,5 mm diameter glass beads (Sartorius). Then the extracts were centrifuged for 5 min at 4 °C at maximum speed, 300 µL of the supernatant were transferred to a new tube, and 20 µL were separated in a new tube as input.

To co-immunoprecipitate the desired proteins, 50 µL of Protein G Dynabeads® were incubated first with 1 µg anti- HA High Affinity antibody (Roche) for 4 hours at 4℃, then the protein extract was added to the beads and incubated O/N at 4℃. Afterwards, Dynabeads®-antibody-proteins complex were washed 3 times using 200 µL RIPA buffer for each wash. Then proteins were eluted by placing the tube on the magnet and removing the supernatant, then the beads were re-suspended in 20µL elution buffer and heated for 10 min at 70 ºC. Finally, the tube was placed again on the magnet and the supernatant was removed and analyzed by Western blotting.

IX. EVALUATION OF CELL DEATH AND OXIDATIVE DAMAGE BY FLOW CYTOMETRY

For the analysis of cellular lysis, oxygen-free radicals (ROS) and variations of the mitochondrial membrane potential (MMP), yeast transformants were grown overnight in 10 mL of SR medium at 30℃. Then cells were sub-cultured in 10 mL SR medium supplemented with galactose 2% (v/v) for 16 h. Afterwards, 1 mL of the culture was taken for each treatment. Rhodamine 123 (5 µg/mL) was added for 2 h. dihydroethidium (2.5µg/mL) for 5 min and propidium iodide (0.004%) for 2 min; samples were diluted 1:10 in PBS to avoid excessive quantity of cells. Three thousand cells were analyzed per second on a FACScan (Becton Dickinson) on the FL2 log scale. WinMDI 2.7 software was used to handle the graphics obtained.

X. Phalloidin staining

The appropriate yeast strain was grown O/N at 30℃ in selective SR medium. The second day cells were refreshed in 10 ml of SR medium to an OD_{600nm} of 0.3 and incubated for 4 hours at 30℃. Then cells were fixed by adding 1 ml of formaldehyde

directly to the culture medium to a final concentration of 2-4% (from a commercial 37% liquid stock) and then incubated for 1 h at 4 ℃. Afterwards, cells were harvested by spinning at 3000 rpm for 2 min, washed three times with buffer 1 [1M KH_2PO_4, 0.1M $MgCl_2$, 0.2M EGTA pH 7.5], and H_2O), and then re-suspended in 100 µL of buffer 1. Fifty µL of 1% Triton X-100 were added to the mix and incubated at room temperature for 2 min. Then cells were washed three times with PBS, re-suspended in 100 µL of PBS, and 10 µL of rhodamine-phalloidin were added and incubated in the dark for 1 h. Then, cells were washed and re-suspended in 100 µL of PBS. Finally, cells were observed under fluorescence microscopy.

XI. BIOINFORMATIC SUPPORT

During the realization of this thesis, the following databases and bioinformatics tools were used:

Pubmed (PMC). Digital repository of scholarly articles that have been published within the biomedical and life sciences journal literature developed by the National Center for Biotechnology Information. http://www.ncbi.nlm.nih.gov/pubmed

NCBI Conserved Domain Database (CDD) https://www.ncbi.nlm.nih.gov/cdd/

***Saccharomyces* Genome Database (SGD).** Server of Stanford University with access to different tools for *S. cerevisiae* sequence analysis, projects of functional genomics, literature review, etc. http://www.yeastgenome.org/

Basic Local Alignment Search Tool (BLAST). Enables a researcher to compare a query sequence with a library of sequences. http://www.ncbi.nlm.nih.gov/blast/Blast.cgi

ClustalW. Used to detect similarities and differences between two or more sequences. http://embnet.vital-it.ch/software/ClustalW.html

Psi-blast based secondary structure prediction (PSIPRED). Permit to predict protein secondary structure. http://bioinf.cs.ucl.ac.uk/psipred/

RESULTS

I. STUDY OF THE EFFECT OF THE EXPRESSION OF SteA IN *Saccharomyces cerevisiae*

I.1. The SteA protein is unique to the *Salmonella* genus

SteA is among a group of 9 *Salmonella enterica* serovar Typhimurium effector proteins that can be translocated by both the SPI-1 and the SPI-2 T3SSs (Geddes *et al.*, 2005; Cardenal-Muñoz & Ramos-Morales, 2011). It is a short protein of 210 amino acids that does not display any known conserved domains according to CDD (NCBI Conserved Domain Database).

We decided to check the similarity of SteA sequence of *S.* Typhimurium with other proteins deposited in databases by using the basic local alignment search tool BLAST-P (NCBI). The results showed high conservation of the SteA sequence (more than 85% identity) among *S. enterica* subsp. *enterica* serovars (Fig. 13). SteA sequence is also conserved in *S. enterica* subsp. *arizonae* and *S. bongori* but with only 34.62% and 37.5% of identity compared with *S. enterica* subsp. *enterica* serovar Typhimurium, respectively. This does not only reflect a lower identity, but also the fact that SteA is a larger protein in these *Salmonellae*. However, no significant similarity was found with proteins from other *Enterobacteriaceae* or any other bacterial species. This indicates that SteA is rather specific for the genus *Salmonella* (Fig. 13).

I.2. Expression of SteA in *S. cerevisiae* impairs cell growth

Our group previously reported the isolation of a fragment of the *S. enterica* subsp. *enterica* serovar Typhi *steA* gene in a screen aimed to unveil *Salmonella* genes that inhibited yeast growth when overexpressed (Alemán *et al*., 2009). Due to the high similarity found between SteA in *S.* Typhi and *S.* Typhimurium we decided to study whether SteA of *S.* Typhimurium also interfered with yeast growth. We developed a GST-SteA fusion to be expressed from the strong galactose-regulatable *GAL1* promoter in *S. cerevisiae*. As shown in figure 14, overexpression of GST-SteA in yeast led to growth inhibition at both 28 and 37 ºC incubation temperatures. Nevertheless, only severe overexpression led to toxicity, because the observation of this phenotype required double selection in medium lacking both uracil and leucine, which forces a high number of copies of the *URA3 leu2-d* pEG(KG) expression plasmid (Mitchell *et*

al., 1993). Thus, SteA of *S*. Typhimurium causes toxicity in yeast when highly overproduced.

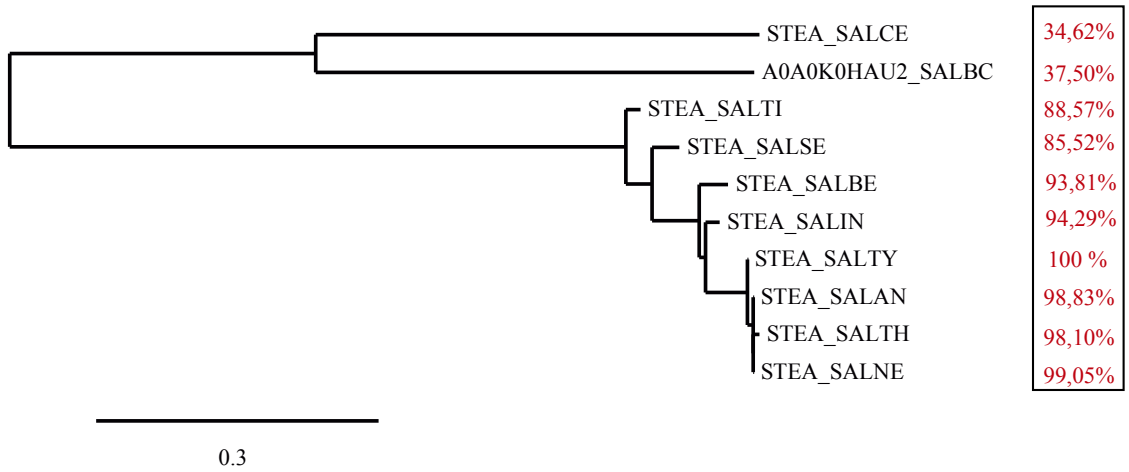

Figure 13. Phylogenetic tree of SteA like proteins.
Dendrogram of the full amino acid sequences of SteA in *S. enterica* subsp. *enterica* serovar Typhimurium (SALTY) and their orthologues in different species, subspecies and serovars. *S*. Senftenberg (SALSE), *S enterica* subsp. *enterica* serovar. Typhi (SALTI), *S. enterica* subsp. *arizonae* (SALCE), *S. bongori* (SALBC), *S. enterica* subsp. *enterica* serovar Berta (SALBE), *S. enterica* subsp. *enterica* serovar Infantis (SALIN), *S. enterica* subsp. *enterica* serovar Thompson (SALTH), *S. enterica* subsp. *enterica* serovar Anatum (SALAN), and *S. enterica* subsp. *enterica* serovar Newport (SALNE). The box at the right shows the percentage of identity between the full amino acid sequence of each SteA orthologue and that of SteA from *S. enterica subsp. enterica* serovar Typhimurium (SALTY).

I.3. The first 97 amino acids of SteA are necessary and sufficient to induce toxicity in *S. cerevisiae*

Since full length SteA is toxic, we decided to check whether a specific region of the protein was responsible for the growth inhibitory effect. In the absence of further structural clues, we chose to split the protein in two halves between amino acids 97 and 98, thus intending not to distort putative secondary structures, as this site seems to map between two β-strands downstream a predicted α-helix in the secondary structure, as deduced by PSIPRED analysis (Fig. 14). The first 97 amino acids were sufficient to induce toxicity at 28 ºC, although with a reduced efficiency as compared to full-length SteA. In contrast, the GST-SteA$^{98\text{-}210}$ truncated version did not cause toxicity at any temperature, as compared to GST alone (Fig. 15). Therefore the N-terminal region

comprising the first 97 amino acids of SteA is necessary and sufficient to impair yeast growth when overexpressed.

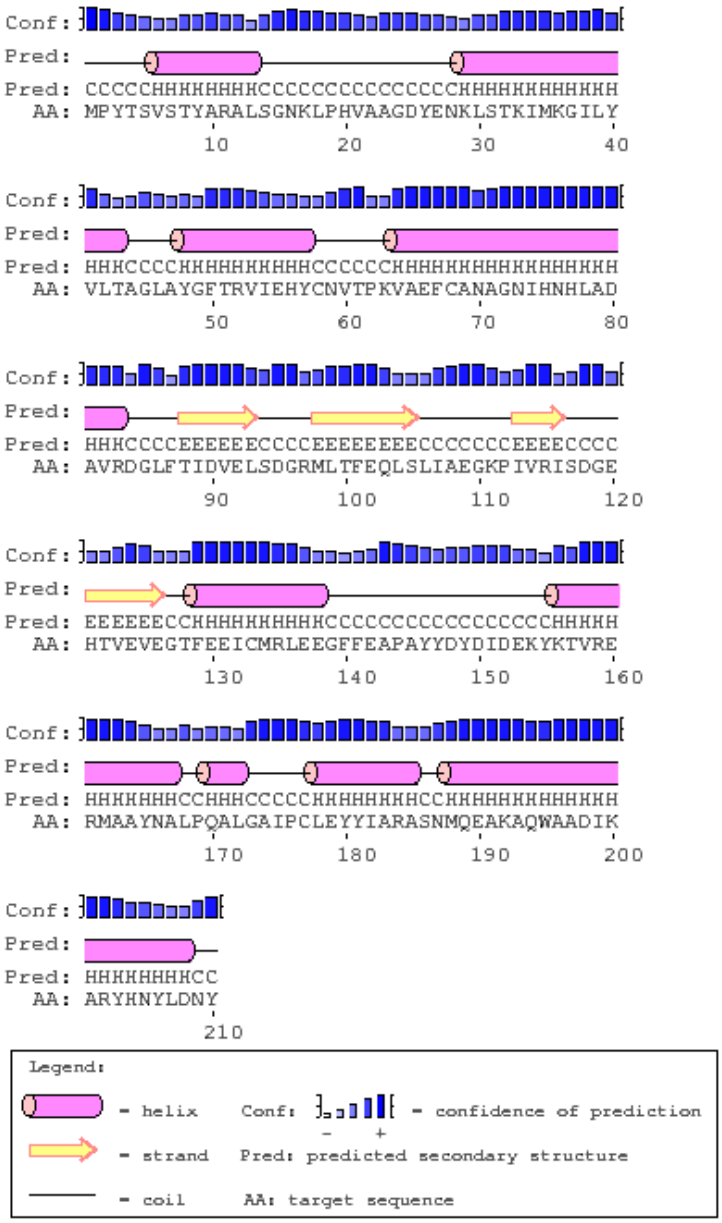

Figure 14. PSI-PRED secondary structure prediction for the SteA sequence.
The predicted secondary structure of *Salmonella* Typhimurium effector protein SteA from PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred) is depicted. The blue bars for each amino acid represents the confidence of each prediction. The taller the bar, the higher the confidence.

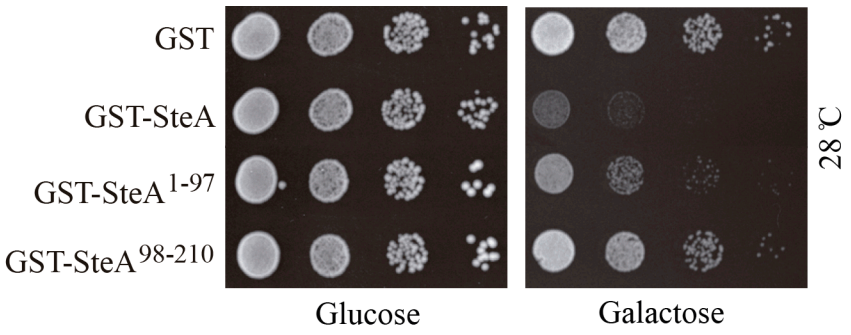

Figure 15. Effect of overexpression of full length or truncated GST-SteA versions on *S. cerevisiae* growth. Ten-fold serial dilutions of cells (YPH499) expressing either GST, full length GST-SteA or truncated halves, GST-SteA$^{1-97}$ and GST-SteA$^{98-210}$ from pEG(KG)-based plasmids, were spotted and incubated for 72 h in solid SC(-Ura-Leu) agar supplemented with glucose (repression conditions) or galactose (induction conditions) at 28℃.

I.4. SteA causes mitochondrial condensation in *S. cerevisiae*

A number of pathogenic bacteria effectors target mitochondria to modulate the host apoptotic machinery, as the vacuolating cytotoxin A (VacA) of *Helicobacter pylori* that engages the mitochondrial fission machinery to induce host cell death (Jain *et al.,* 2011). Thus, we wanted to check if the toxicity of SteA might be related to significant changes in the mitochondrial morphology when expressed in yeast.

As shown in figure 16A, *S. cerevisiae* cells co-expressing either full length SteA or the truncated version SteA$^{1-97}$ with the fluorescent mitochondrial acetohydroxyacid synthase Ilv6-DsCherry as a marker for this organelle (Huh *et al*., 2003), showed altered mitochondrial morphology. Instead of several normal elongated mitochondria per cell, SteA and SteA$^{1-97}$-expressing cells usually showed large condensed mitochondrial compartments. In agreement with the higher toxicity displayed by full-length SteA, the percentage of cells showing condensed mitochondria was higher in cells expressing SteA than in those expressing the N-terminal region (Fig. 16B). In contrast, yeast expressing the SteA$^{98-210}$ version did not show any significant increase in the percentage of cells showing abnormal mitochondria (Fig. 16B). Thus, SteA causes mitochondrial condensation when overproduced in yeast, and its amino-terminal half is necessary and sufficient to induce this effect.

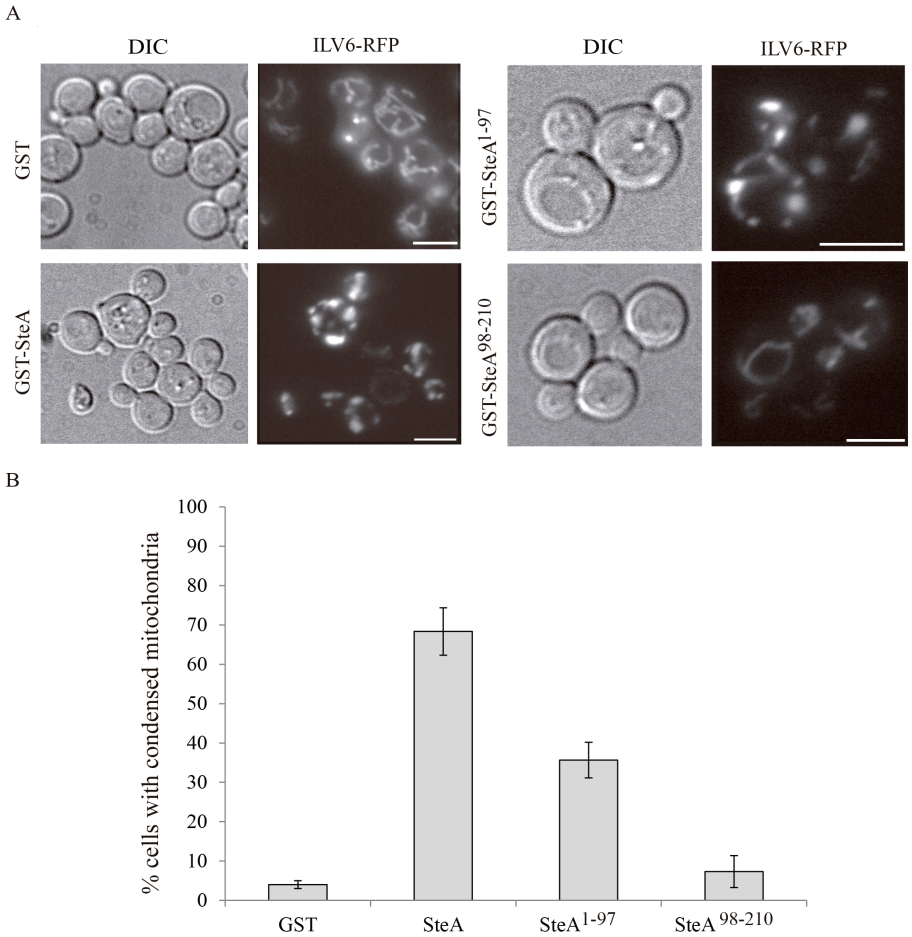

Figure 16. Mitochondrial morphology in yeast cells expressing full length SteA or its truncated versions.
Differential Interference Contrast (DIC) microscopy and fluorescence images of the same fields showing cell morphology (left panels) and localization of ILV6-RFP (expressed from pILV6) (right panels) of *S. cerevisiae* YPH499 expressing GST, full length or truncated GST-SteA from pEG(KG)-based plasmid after 4h incubation in SC-R (-Ura-Leu-Trp) media containing 2% galactose (w/v). All scale bars indicate 5µm. (**B**) Quantification of the percentage of cells with condensed mitochondria. Bars in graphs indicate the standard deviation from three different clones; at least 100 cells were counted for each clone.

I.5. Expression of SteA in *S. cerevisiae* does not alter the morphology of the endoplasmic reticulum

Since the mitochondria and the endoplasmic reticulum (ER) connect together at multiple contact sites to form specific domains, termed mitochondria-ER associated membranes (MAMs) (Marchi *et al.*, 2014), we thought that SteA might also be affecting the ER morphology. In order to test this, full length SteA, as well as truncated versions of SteA were co-expressed in yeast cells with the fluorescent ER marker HDEL-DsCherry (that localizes at the perinuclear and peripheral ER). As revealed by fluorescence microscopy, SteA did not cause ER morphological alterations when

overproduced in yeast (Fig. 17).

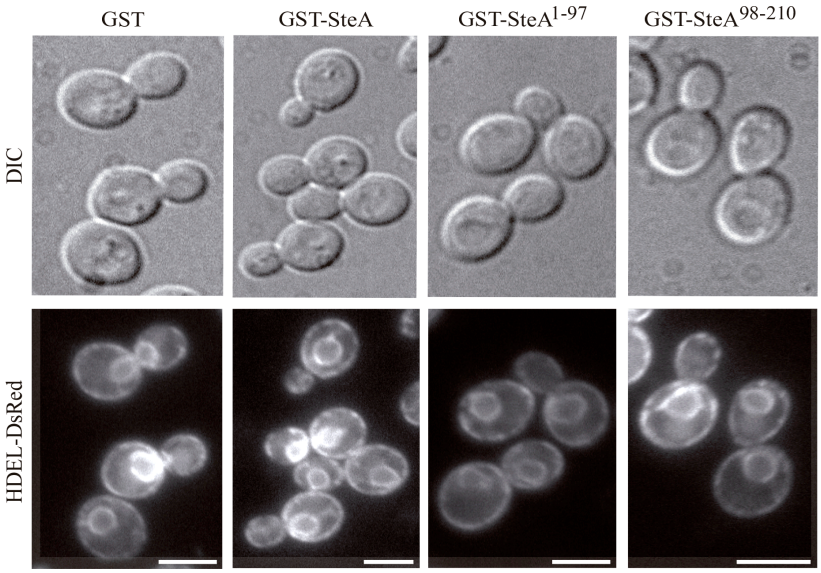

Figure 17. Endoplasmic reticulum morphology in yeast cells expressing full length or truncated GST-SteA versions.
Differential Interference Contrast (DIC) microscopy and fluorescence images showing cell morphology (left panels) and localization of HDEL-DsRed (right panels). VHY87 cells harboring the HDEL-DsRed were transformed with pEG(KG)-based plasmid expressing either GST, full length GST-SteA or truncated versions (GST-SteA$^{1-97}$and GST-SteA$^{98-210}$) as indicated. Cells were grown in SC-R (-Ura) and induced by the addition of galactose to 2% (w/v) followed by incubation for 4h. All scale bars indicate 5µm.

I.6. Expression on SteA in yeast does not disorganize the actin cytoskeleton

The actin cytoskeleton is a dynamic structure that participates in cellular functions including the maintenance of cell morphology and polarity (Anesti & Scorrano, 2006). In yeast, interactions between mitochondria and the cytoskeleton are essential for normal mitochondrial morphology, motility and distribution (Boldogh & Pon, 2006). Therefore, we examined the effect of SteA expression in *S. cerevisiae* on the actin cytoskeleton.

S. cerevisiae expressing either the GST alone or full-length GST-SteA and stained with fluorochrome-conjugated phalloidin showed a normal distribution of actin,

with actin cables extending along the mother-daughter cell axis, and cortical patches showing a marked polarized distribution in nascent buds (Fig. 18). Therefore, SteA does not affect the actin cytoskeleton in yeast cells.

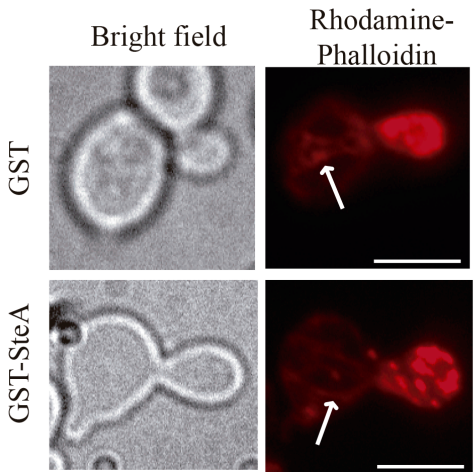

Figure 18. Actin cytoskeleton morphology in yeast cells expressing SteA
Bright field microscopy and fluorescence images showing cell morphology (left panels) and actin staining (right panels). YPH499 strain was transformed with pEG(KG)-based plasmid expressing either GST or full length GST-SteA, and cells were grown in SC-R (-Ura-Leu-Trp), induced by the addition of galactose to 2% (w/v) incubated for 4h, and then stained with rhodamine phalloidin. All scale bars indicate 5µm.

I.7. SteA does not affect the mitochondrial outer membrane potential when expressed in yeast

In order to determine the molecular mechanism behind the toxicity of SteA in yeast, we decided to test whether mitochondrial membrane potential (MMP) which is a marker related to programmed cell death (Madeo *et al*., 2007) was affected upon overexpression of SteA in *S. cerevisiae.* To this end, cells were transformed with plasmids expressing GST, GST-SteA, GST-SteA$^{1-97}$ or GST-SteA$^{98-210}$ and were incubated in the presence of rhodamine 123 as a fluorescent marker for loss of MMP. No significant variation in the percentage of rhodamine-positive cells was observed in any of the cultures (Fig. 19). Thus, in spite of severely affecting mitochondrial morphology, SteA does not affect the MMP when overexpressed in yeast.

I.8. SteA overexpression does not lead to the production of reactive oxygen species in yeast

Since cell death or growth impairment are associated in many cases with intracellular production of reactive oxygen species (ROS) (Perrone *et al*., 2008), we decided to check if the overexpression of this effector caused the formation of ROS. For this purpose, yeast cells expressing GST, GST-SteA, GST-SteA$^{1-97}$ or GST-SteA$^{98-210}$ were treated with dihydroethidium (DHE). The superoxide-driven conversion of non-fluorescent DHE into fluorescent ethidium can be readily monitored by flow cytometry. Compared to cells expressing GST alone, which showed a percentage of positive cells of 1,61%, none of the transformants expressing any of the SteA versions tested showed a significant increase in the percentage of cells above the gating threshold (Fig. 20). Therefore SteA does not lead to the production of ROS, and the toxicity caused by its overexpression cannot be related to oxidative damage by these reactive species.

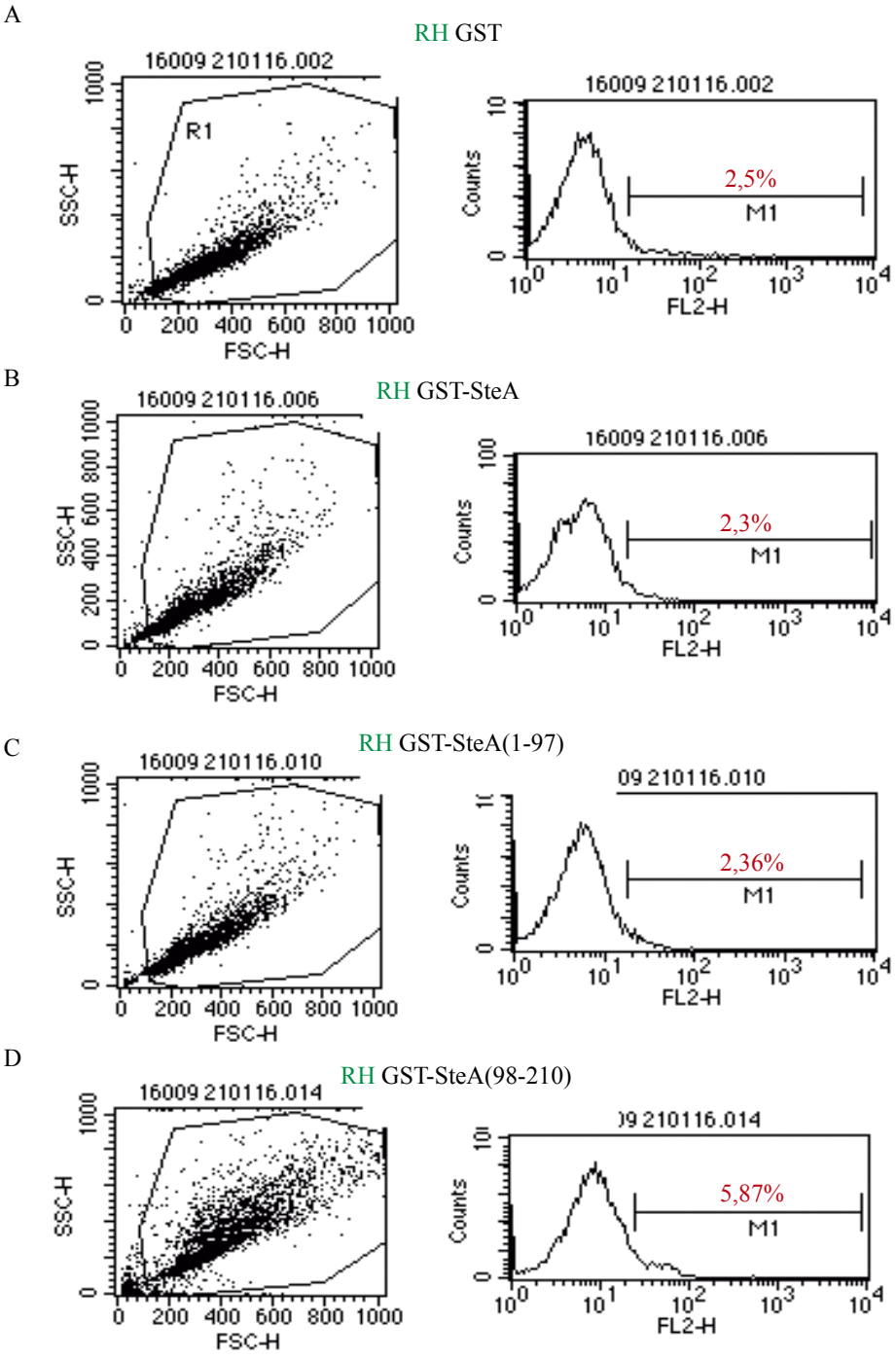

Figure 19. Study of the mitochondrial membrane potential in yeast cells expressing SteA.
Flow cytometric analyses on yeast strain YPH499 (**A**) expressing GST alone, (**B**) full length GST-SteA or (**C**, **D**) the indicated truncated versions of GST-SteA, all from pEG(KG)-based plasmids. Cells were incubated O/N in SC-R (-Ura -Leu) and then were induced for 16 h in SC-R (-Ura -Leu) supplemented with 2% galactose (w/v), and dihydroethidium (DHE) (2.5 µg/ml) was added to the suspension for 5 min at 30℃. Then cells were diluted 1:10 in PBS and analyzed. M2 indicates the fluorescence positive interval over the gating threshold and the percentage of cells beyond the threshold is shown in red in each graphic (right panels).

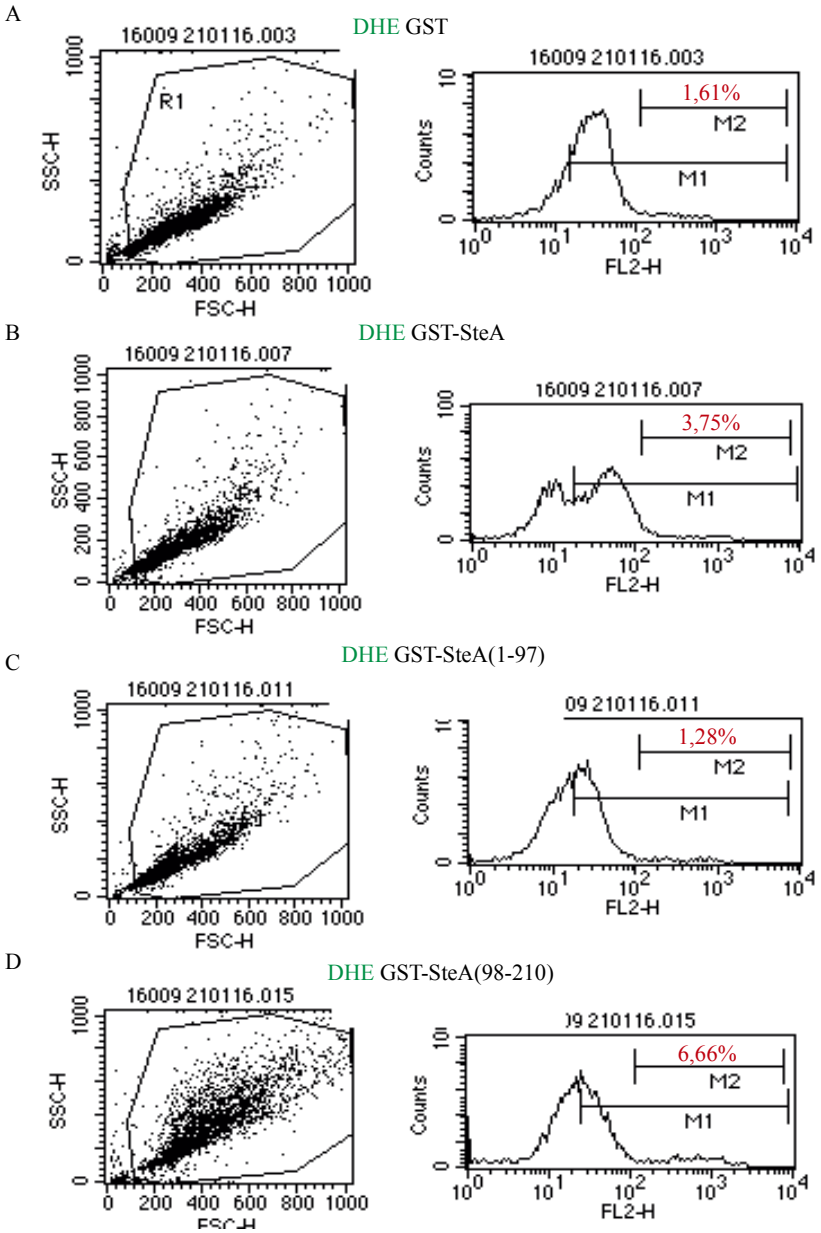

Figure 20. Detection of reactive oxygen species (ROS) in yeast cells expressing SteA.
Flow cytometric analyses on yeast strain YPH499 (**A**) expressing GST alone, (**B**) full length GST-SteA or (**C**, **D**) the indicated truncated versions of GST-SteA, all from pEG(KG)-based plasmids. Cells were incubated O/N in SC-R (-Ura -Leu) and then were induced for 16 h in SC-R (-Ura -Leu) supplemented with 2% galactose (w/v), and dihydroethidium (DHE) (2.5 µg/ml) was added to the suspension for 5 min at 30℃. Then cells were diluted 1:10 in PBS and analyzed. M2 indicates the fluorescence positive interval over the gating threshold and the percentage of cells beyond the threshold is shown in red in each graphic (right panels).

I.9. SteA does not affect the selective permeability of the plasma membrane when expressed in *S. cerevisiae*

Necrotic cell death is characterized by a plasma membrane rupture (Eisenberg *et al.,* 2010) that can be detected by staining with propidium iodide (PI), a nucleic acid-binding red-fluorescent compound not permeant to living cells. During apoptosis, the cell membrane can suffer many changes including a gradual increase in permeability that may eventually lead to secondary necrosis (Nelson *et al*., 2011). To further investigate SteA toxicity, we decided to check the loss of selective permeability of the plasma membrane by treating cells expressing GST, GST-SteA^{WT}, GST-SteA$^{1-97}$ or GST-SteA$^{98-210}$ with this dye. None of the transformants expressing SteA or its truncated versions compared to cells expressing GST alone showed a significant increase in the percentage of PI-positive cells (Fig. 21). All together, these results indicate that overexpression of SteA in yeast, in spite of causing an altered mitochondrial morphology, does not lead to MMP loss or mitochondrial dysfunction-related oxidative damage, and that growth inhibition is not related to enhanced cell death either by apoptosis or necrosis.

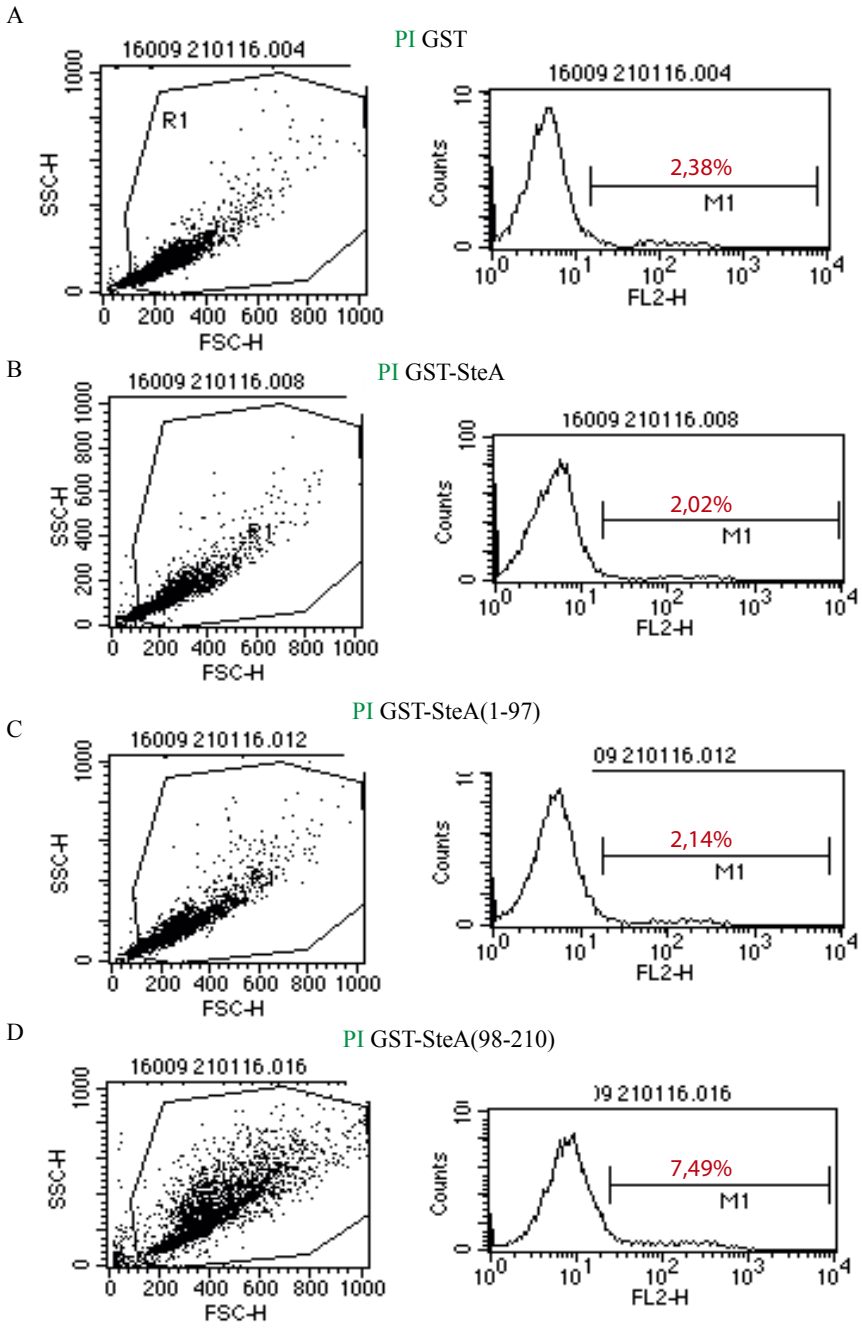

Figure 21. Analysis of plasma membrane permeability in yeast cells expressing SteA.
Flow cytometric analyses on yeast strain YPH499 (**A**) expressing GST alone, (**B**) full-length GST-SteA or (**C**, **D**) the indicated truncated versions of GST-SteA, all from pEG(KG)-based plasmids. Cells were incubated O/N in SC-R (-Ura -Leu) and then were induced for 16 h in SC-R (-Ura -Leu) supplemented with 2% galactose (w/v), and propidium iodide (PI) (0.05 mg/ml) was added to the suspension for 2 min at 30 ℃. Then cells were diluted 1:10 in PBS and analyzed. M1 indicates the fluorescence positive interval over the gating threshold and the percentage of cells beyond the threshold is shown in red in each graphic (right panels).

I.10. SteA does not affect the vacuolar morphology in *S. cerevisiae*

To assess the effect of SteA on vacuolar morphology, cells were transformed with pEG(KG) plasmids expressing GST and GST-SteA, and the vacuoles were stained with the 7-amino-4-chloromethylcoumarin (CMAC) which accumulates in the vacuolar lumen. CMAC fluorescence relies on the acid pH of this compartment. Then cells were visualized under fluorescence microscopy. All cells showed a normal vacuolar staining, suggesting a normal vacuolar morphology (Fig. 22). Therefore, SteA toxicity in *S. cerevisiae* is not related to apparent changes in vacuolar morphology or maturation.

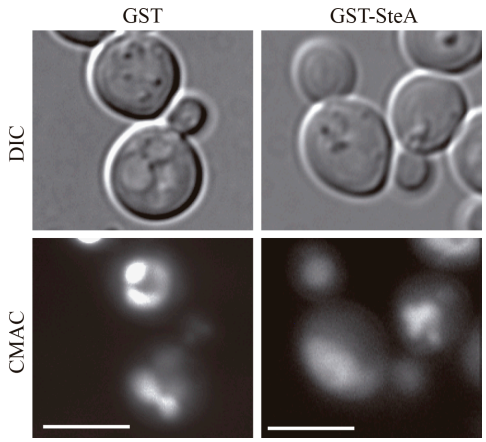

Figure 22. CMAC vacuole staining in *S. cerevisiae* expressing SteA.
Differential Interference contrast (DIC) and fluorescence images microscopy showing cell morphology (DIC; upper panels) and CMAC vacuolar lumen staining (lower panels) in *S. cerevisiae* YPH499 strain transformed with pEG(KG)-based plasmids expressing either GST or GST-SteA, after 4 h of incubation in SC-R (-Ura -Leu) medium containing 2% galactose (w/v). Cells were stained adding CMAC at 0.6 µM, and incubated 5 min at 25℃. All scale bars indicate 5 µm.

II. STUDY OF THE LOCALIZATION OF SteA IN *S. cerevisiae*

II.1. SteA is associated with the host cell plasma membrane and vacuole when expressed in *S. cerevisiae*

In order to study SteA localization *in vivo*, we developed both GFP-SteA^{WT} (GFP fused to the N-terminus of SteA) and SteA^{WT}-GFP (GFP fused to the C-terminus of SteA) for expression in wild-type *S. cerevisiae*. Interestingly, both fusions localized at yeast plasma membrane (PM) and vacuoles. In vacuoles, GFP-SteA localized at the lumen but also concentrated more conspicuously at the vacuolar membrane, especially at vacuole-to-vacuole contact areas (Fig. 23A and C); whereas SteA-GFP mainly decorated the vacuolar lumen (Fig. 23B). Vacuolar localization was confirmed by co-staining cells expressing SteA-GFP with FM4-64 [N-(3-triethylammoniumpropyl)-4-(p-diethylaminophenyl-hexatrienyl) pyridinium dibromide], an endocytic marker that marks the vacuole membrane (Fig. 23B). PM localization was confirmed by co-expression of GFP-SteA with Lact-C2-mCherry (the lactadherin C2 domain binds phosphatidylserine at the PM), which showed a co-localization of both proteins (Fig. 23C). Overall these results reveal the association of SteA to the plasma and vacuolar membranes as a feature of this effector when expressed in yeast, which can provide important clues for its behavior in the host cell.

II.2. The amino-terminal region of SteA localizes to the ER when expressed in *S. cerevisiae*

Since the amino-terminal half of SteA is responsible and sufficient for toxicity, we decided to check the localization of a SteA$^{1-97}$-GFP fusion protein. Expressed in yeast, SteA$^{1-97}$-GFP had a different localization in the cell, faintly marking the PM and the nuclear periphery, suggesting localization at the ER. This was confirmed by co-staining nuclei with DAPI (Fig. 24A) and with the ER marker HDEL-DsRed. As shown in figure 24B the amino-terminal region of SteA localized to both yeast perinuclear and peripheral ER. This result implies that important determinants for the localization of SteA to the PM and the vacuole lie in the carboxi-terminal half of the protein.

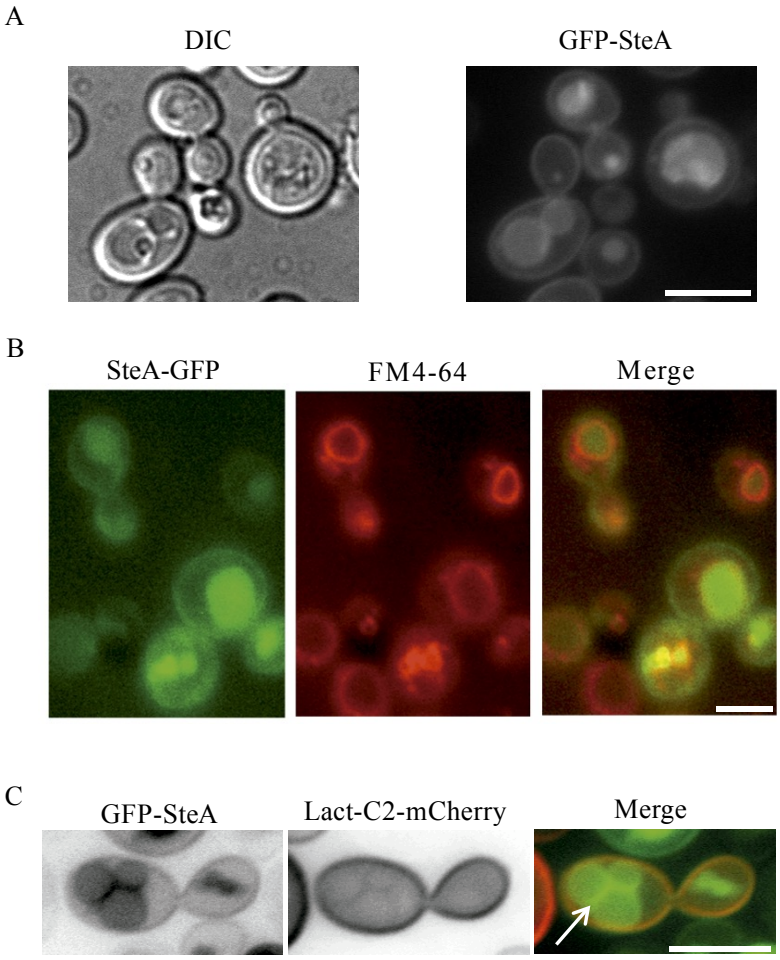

Figure 23. Plasma membrane and vacuolar localization of SteA in *S. cerevisiae*.
(A) Differential Interference Contrast (DIC) microscopy and fluorescence images showing cell morphology (left panel) and localization of GFP-SteA (right panel) of *S. cerevisiae* YPH499 transformed with pYES3GFP-SteA after 4 h incubation in SC-R (-Leu -Trp) medium containing 2% galactose. (**B**) Fluorescence microscopy images showing SteA-GFP expressed in *S. cerevisiae* YPH499 after induction in media containing galactose for 4 h and treatment for 1 h with the endocytic marker FM4-64 (to counterstain the vacuolar membrane; red). (**C**). Fluorescence microscopy images showing GFP-SteA (pYES3GFP-SteA) localization (inverted in single channel to black and white image; green in merged image) and Lact-C2-mCherry (inverted in single channel to black and white image; red in merged image) of *S. cerevisiae* YPH499 transformed with pYES3GFP-SteA after 4 h of induction in medium containing galactose.

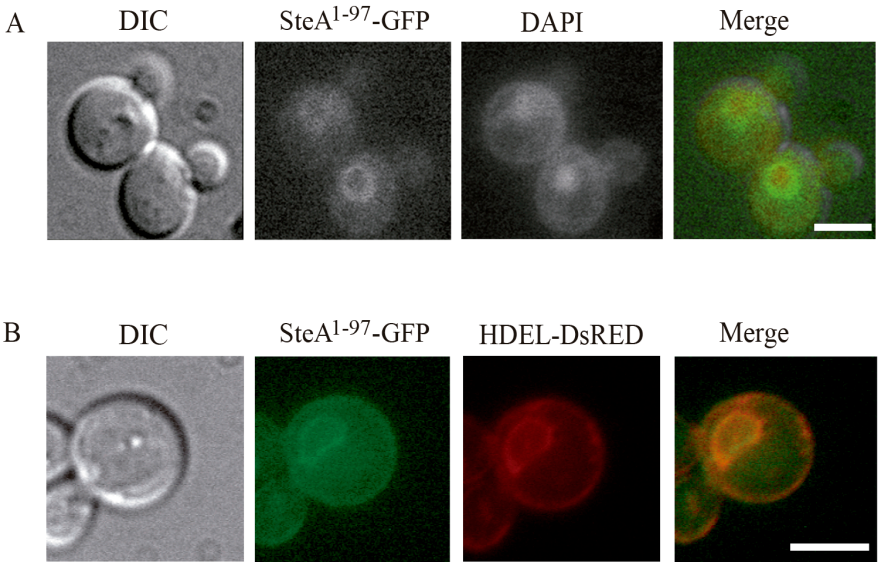

Figure 24. ER localization of SteA$^{1-97}$-GFP in *S. cerevisiae.*
(A). Localization of SteA$^{1-97}$-GFP (YCpLG-SteA$^{1-97}$-GFP). Cells were grown in SC-R (-Leu) and induced by the addition of galactose to 2% (w/v) followed by incubation for 4 h and then counterstained with DAPI to allow visualization of the nuclei (DAPI is artificially colored in red to improve contrast) (**B**). VHY87 strain cells harboring the HDEL-DsRED were transformed with the YCpLG plasmid expressing SteA$^{1-97}$-GFP and cells were grown in SC-R (-Leu) and induced by the addition of galactose to 2% (w/v) followed by incubation for 4 h. All scale bars indicate 5 µm.

II.3. SteA localization is not actin-dependent

In order to check whether the localization of SteA was dependent on actin function, GFP-SteA was expressed in *S. cerevisiae* and cells were treated with the actin filament disrupting compound latrunculin B. GFP-SteA did not show any change in either PM or vacuolar localization in cells exposed to latrunculin B as compared with control untreated cells. The fact that the localization of SteA is preserved after depolymerization of cellular actin suggests that the actin cytoskeleton is not required for recognition of cellular membranes by SteA (Fig. 25).

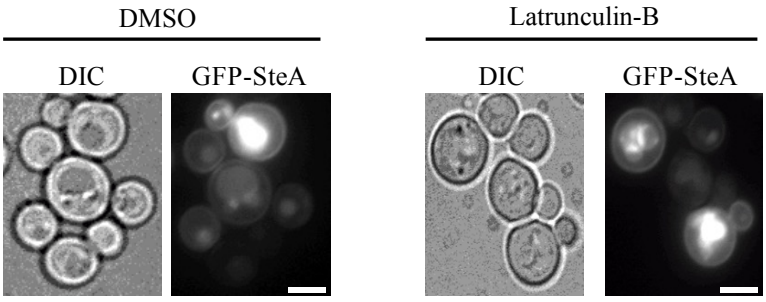

Figure.25 SteA localization in yeast cells treated with latrunculin B.
Differential Interference Contrast (DIC) microscopy (left panels) and fluorescence images (right panels) respectively showing the localization of GFP-SteA in *S. cerevisiae* YPH499 cells transformed with pYES3GFP-SteA. Cells were grown in SC-R (-Leu) and induced by the addition of galactose to 2% (w/v) for 4 h and treated for 1 h with latrunculin B (0,5 µg/mL) (right panels) or DMSO (left panels). All scale bars indicate 5 µm.

II.4. SteA plasma membrane localization in yeast requires the phosphoinositide 4-kinase Stt4

Phosphoinositides are key markers for specific cellular membranes in eukaryotic cells. PI(3)P tags endocytic and vacuolar compartments, the PM is typically marked by PI(4,5)P_2, and PI(4)P is characteristic of Golgi membranes and acts as a precursor of PI(4,5)P_2 at the PM (Dickson *et al.*, 2014). In *S. cerevisiae*, most PI(4)P is generated by two phosphatidylinositol 4-kinases (PI4Ks) with distinct subcellular localizations (De Matteis *et al.*, 2013) that produce distinct pools of this phosphoinositide on different intracellular membranes. Pik1 localizes at the Golgi and accounts for 45% of the cellular PI(4)P, and Stt4 localizes at the PM and supports the production of 40% of PI(4)P. A third kinase, Lsb6, accounts for the remaining PI(4)P cellular content (Audhya *et al.*, 2000) (Fig. 26A). Yeast constitutes an attractive experimental model to study the possible involvement of spatially regulated phosphoinositide pools such as those of PI(4)P in the subcellular localization of SteA in eukaryotic cells because, although *PIK1* and *STT4* genes are essential, we can use temperature-sensitive (*ts*) conditional mutants in which most of the cellular PI(4)P would be either at the PM (*pik1-ts* mutant) or at the Golgi (*stt4-ts* mutant) at the restrictive temperature. In addition, we used as well a *lsb6*Δ mutant, since *LSB6,* contrary to *STT4* and *PIK1*, is not an essential gene.

To this end, we expressed GFP-SteA in *S. cerevisiae stt4-ts* mutant and *lsb6*Δ mutant backgrounds and SteA-GFP in a *pik1-ts* mutant background. We used SteA-GFP

and GFP-SteA in the different yeast mutants to account for the markers available for transformation in the respective strains. Fluorescence microscopy of GFP-SteA (Fig. 26B), SteA-GFP (Fig. 26C) and GFP-SteA (Fig. 26D) in these mutant backgrounds revealed that inactivation of Stt4 consistently led to disappearance of the PM pool of GFP-SteA, whereas the vacuolar localization was not affected. Only 9.6% of *stt4-ts* mutant cells at the restrictive temperature showed GFP-SteA located at the PM (Fig. 26E). In contrast, neither inactivation of Pik1-Ts at 37 ºC nor the absence of Lsb6 affected the subcellular localization of SteA-GFP and GFP-SteA respectively, as compared to wild type cells (Fig. 26C and D). Therefore, the localization of SteA at the yeast PM is dependent on the activity of the PM-localized Stt4 but not of the Golgi-localized Pik1 nor minor Lsb6, suggesting that PI(4)P might be critical for targeting SteA to the PM.

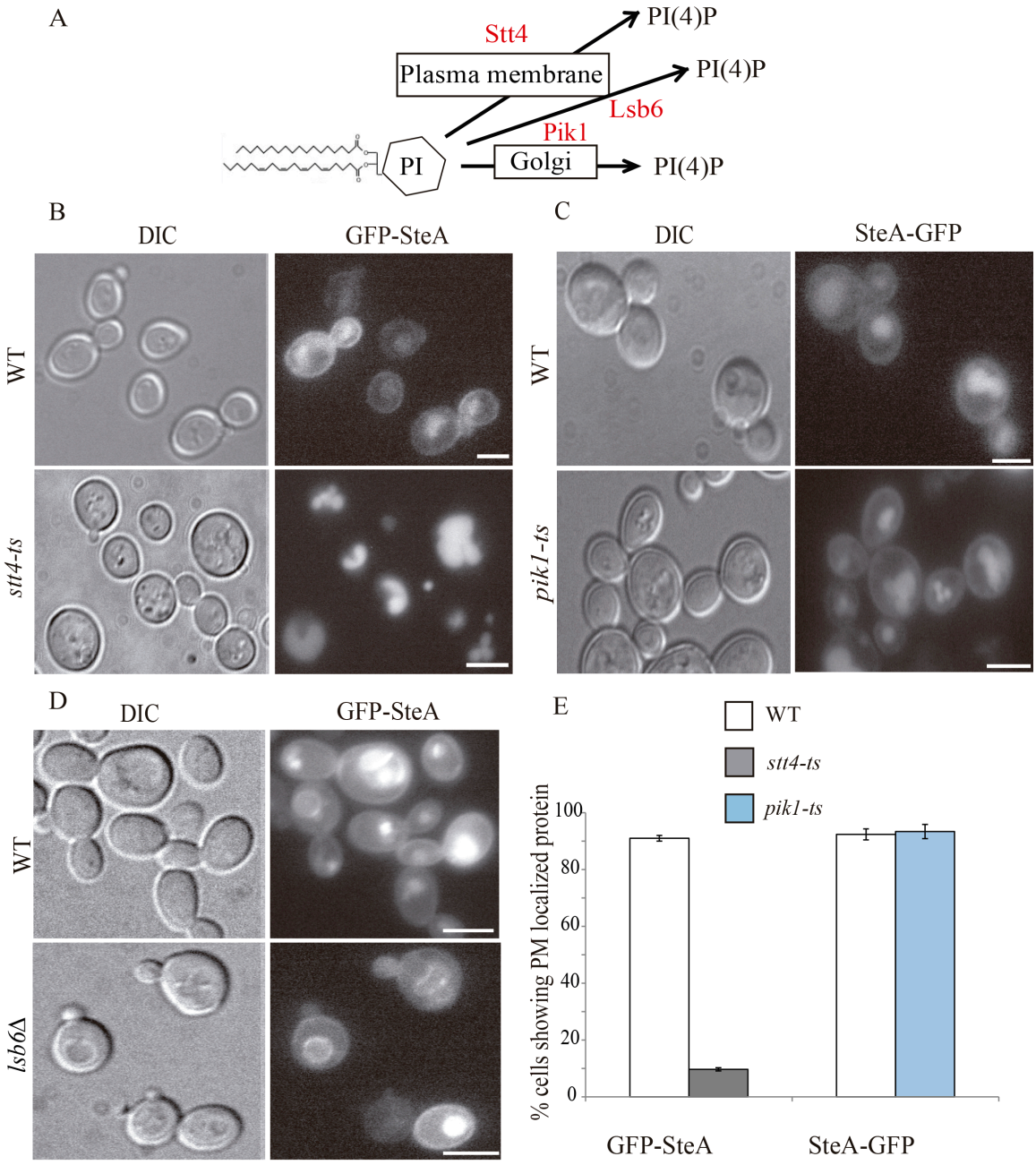

Figure 26. Effect of PI(4)P depletion on SteA localization

(A) Scheme of the activity of *S. cerevisiae* PI 4-kinases Pik1, Stt4 and Lsb6, and the subcellular membranes in which they function. Differential contrast microscopy (DIC; left panels) and fluorescence images (right panels) of (**B**) *S. cerevisiae* YPH499 (WT) or YFR201 (isogenic *stt4-ts*) strains, grown at 24ºC after GFP-SteA induction from plasmid pYES3GFPSteA in SR (-Trp) containing 2% galactose for 4 h and then shifted to 37°C for 1 h; (**C**) *S. cerevisiae* YPH499 (WT) or YES95 (*pik1-ts*) strains expressing SteA-GFP from plasmid YCpLG-SteAGFP cultured as in B; (**D**) *S. cerevisiae* BY4741 (WT) or Y01323 (*lsb6Δ::KanX4)* expressing GFP-SteA from plasmid pYES2GFP-SteA, after 4 hours of incubation in SR (-Ura) media containing 2% galactose. Scale bars indicate 5 µm. (**E**) Percentage of cells showing plasma membrane GFP fluorescence processed as in B, C and D. Bars indicate the standard deviation from three different clones; at least 100 cells were counted for each clone.

II.5. PI(4, 5)P_2 is not crucial for SteA plasma membrane localization

PI(4)P at the yeast PM is the precursor of PI(4,5)P_2, a typical marker of the PM (Strahl & Thorner, 2007), synthesized *in situ* by the PI(4)P 5-kinase Mss4 (Fig. 27A). Therefore, we hypothesized that maybe the lack of PI(4,5)P_2 at the PM as a consequence of PI(4)P depletion, rather than the lack of PI(4)P on itself, was the cause for the absence of association of GFP-SteA with the PM in the *stt4-ts* mutant. To test this hypothesis, we expressed GFP-SteA in a *S. cerevisiae mss4-ts* mutant. We confirmed that when this mutant expressed the PI(4,5)P_2-specific reporter PH(PLCδ)-GFP (Stefan *et al.*, 2002), the PM signal observed at permissive temperature (28ºC) was lost at the restrictive temperature (37°C) (Fig. 27C). In contrast, GFP-SteA still localized at the PM when expressed in *mss4-ts* mutant cells at the restrictive temperature (Fig. 27B), indicating that the lack of PI(4,5)P_2 at the PM does not alter the localization of GFP-SteA.

We used an alternative approach to eliminate the PI(4,5)P_2 by expressing the mammalian class I phosphatidylinositol 3-kinase (PI3K) catalytic subunit p110α fused to a C-terminal prenylation signal, which fully depletes the PM pool of this phosphoinositide by turning it into PI(3,4,5)P_3 (Rodríguez-Escudero *et al.*, 2005). A catalytically inactive version (p110-KD) was used as a control. As shown in figure 27C, class I PI3K activity did not impair the localization of GFP-SteA at the PM. Overall, these results are consistent with PI(4)P, and not PI(4,5)P_2, being critical for targeting of SteA to the yeast PM.

II.6. SteA localization is independent of endosomal-vacuolar compartment membrane phosphoinositides PI(3)P and PI(3, 5)P_2

PI(3)P and PI(3,5)P_2 are characteristic of yeast endosomes and the vacuole (Fig. 28A). We also wanted to test whether these phosphoinositides were essential for the subcellular localization of SteA. Deletions in the non-essential genes *VPS34*, coding for class III phosphatidylinositol 3-kinase, and *FAB1* coding for the yeast PI(3)P 5-kinase, did not lead to changes in the PM and vacuole localization of SteA (Fig. 28B). Therefore, SteA localization is not dependent on PI(3)P and PI(3,5)P_2.

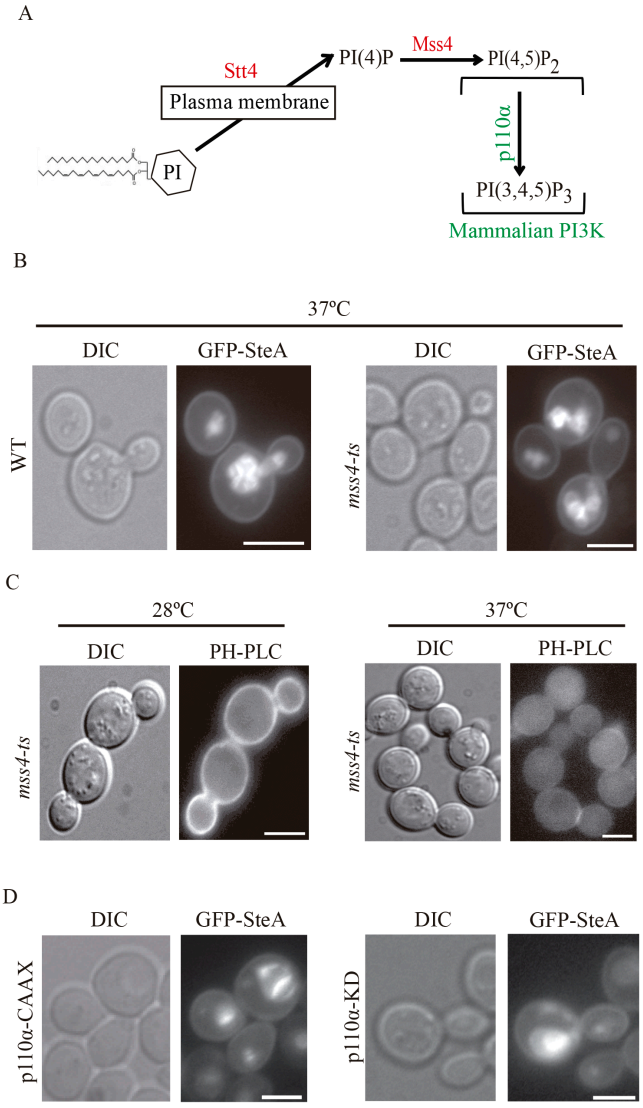

Figure 27. Localization of SteA in yeast cells deficient in PI(4,5)P_2

(**A**) Schematic representation of the activity of relevant yeast *S. cerevisiae* phosphatidyl 4-kinases Stt4, the phosphatidylinositol-4-phosphate 5-kinase Mss4, and the human phosphatidylinositol-4,5-*bis*phosphate 3-kinase PI3K (p110α). (**B**) Differential Interference Contrast (DIC) and fluorescence images showing cell morphology (left panels) and localization of SteA-GFP (right panels) in *S. cerevisiae* YPH499 or SD19-3a (*mss4- ts*) strains, analyzed at the restrictive temperature after induction of GFP-SteA expression from plasmid pYES3GFP in SC-R (-Trp) supplemented with 2% galactose (w/v). (**C**) The SD19-3a (*mss4-ts*) strain was transformed with pRS426GFP-2xPH(PLCδ) (expressing PH-PLC fused to GFP; Stefan *et al*., 2002), incubated in SC-R (-Ura -Trp) with 2% galactose (w/v) for 4 h at 28°C and then shifted to 37°C for 1 h. (**D**) The *S. cerevisiae* YPH499 strain was co-transformed with pYES3-GFP-SteA and plasmids expressing the catalytic subunit of mammalian class I phosphatidylinositol 3-kinase fused to a C-terminal CAAX prenylation signal (WT p110α) or a kinase dead K802R point mutant (p110α-KD). Protein expression in the co-transformants was induced for 6 h in SC-R (-Ura -Trp) supplemented with 2% galactose (w/v). All scale bars indicate 5 µm.

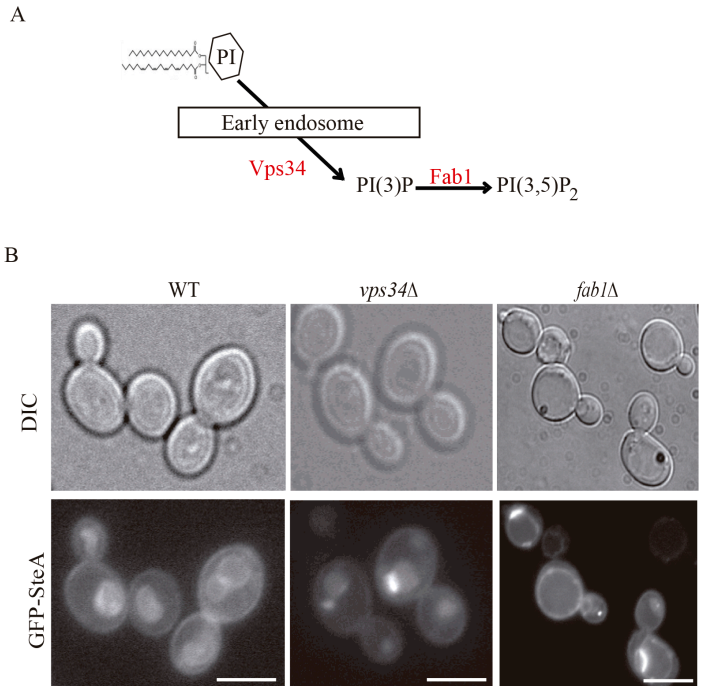

Figure 28. Effect of depletion of both PI(3)P and PI(3,5)P_2 on SteA localization in yeast cells.
(A) Schematic representation of yeast phosphoinositide 3-kinase Vps34, and 1-phosphatidylinositol-3-phosphate 5-kinase Fab1. **(B)** Differential Interference Contrast (DIC) and fluorescence images showing cell morphology (left panel) and localization of GFP-SteA (right panel) in *S. cerevisiae* YPH499 strain (left panel) or in YPH499 isogenic strains carrying a deletion in genes *VPS34* and *FAB1* expressing GFP-SteA from pYES3GFP plasmid, incubated in SC-R (-Trp) containing 2% galactose (w/v) for 4 hours. All scale bars indicate 5µm.

II.7. SteA co-localizes with the PI(4)P-binding domain of the *Legionella pneumophila* effector SidC

The above results suggest that PI(4)P is a major determinant for SteA binding to eukaryotic membranes. The *Legionella pneumophila* effector SidC binds to PI(4)P in mammalian cells (Ragaz *et al*., 2008). This binding activity was mapped to a fragment that does not have any sequence homology with any known PI-binding domain. This fragment, named P4C [PI(4)P-binding of SidC], was suggested to function as PI(4)P-binding probe (Weber *et al*., 2006).

In order to confirm by other strategy that SteA binds PI(4)P at the PM, we decided to co-express the PI(4)P probe P4C with SteA in yeast. SidC (P4C) was cloned into a yeast expression vector fused to the mCherry tag. WT *S. cerevisiae* was

transformed with P4C-mCherry and either GFP- or GFP-SteA-expressing plasmids. P4C localized faintly to the PM and predominantly in intracytoplasmic spots consistent with the Golgi, indicating that yeast reproduces the localization described in mammalian cells (Fig. 29A). It co-localized with SteA at the PM (Fig. 29B) but not in intracytoplasmic membranes. Control cells expressing GFP and P4C-mCherry showed 20% of cells displaying P4C localized at the PM, while cells co-expressing GFP-SteA and P4C-mCherry showed a higher percentage (40%) (Fig. 29C), indicating that SteA-expressing cells have more P4C at the PM. These results, in addition to supporting that SteA binds to PM PI(4)P, hint that SteA expression might lead to enhanced PI(4)P levels at the yeast PM.

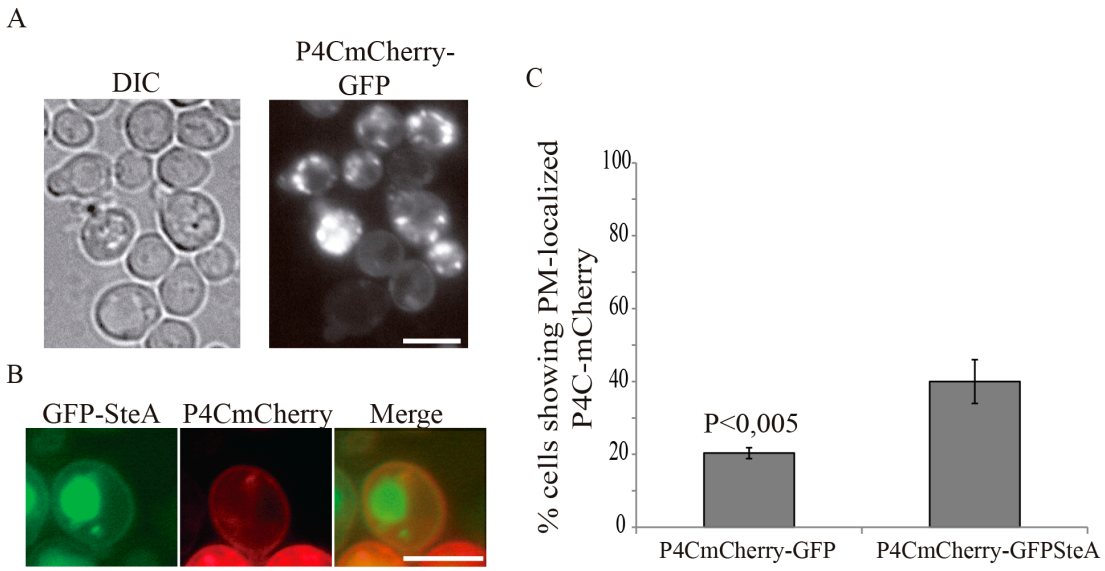

Figure 29. Co-localization of GFP-SteA with the PI(4)P probe SidC(P4C) in yeast cells.
(A) Differential Interference Contrast (DIC) and fluorescence images showing cell morphology (left panel) and localization of P4C-mCherry (right panel) in *S. cerevisiae* YPH499 expressing both GFP from pYES2GFP plasmid and P4C-mCherry from pESC-TRP-mCherry (**B**) Fluorescence microscopy images showing GFP-SteA from plasmid pYES2GFP and P4C-mCherry from plasmid pESC-TRP-mCherry co-expressed in *S. cerevisiae* YPH499 after induction in SC-R (-Ura-Trp) supplemented with 2% galactose (w/v) for 4 h at 30°C. The scale bar indicates 5µm. (**C**) Quantification of the percentage of cells showing SidC(P4C)-mCherry in control cells vs. cells expressing GFP-SteA. Bars in graphs indicate the standard deviation from three different clones; at least 100 cells were counted for each clone.

II.8. Tethering SteA to the plasma membrane when expressed in *Saccharomyces cerevisiae* requires basic residues near its N- terminus

The results above indicate that SteA might bind to the PM by recognition of PI(4)P. To understand how SteA binds to this phosphoinositide we examined the primary structure of SteA to search for known lipid binding motifs, such as phox homology (PX), pleckstrin homology (PH), or phosphotyrosine binding (PTB) domains (Kutateladze, 2010). We could not find any of these motifs, but we found two clusters of positively charged residues, one in its N-terminal half (Lys29, Lys33 and Lys36) and the other in its C-terminal half (Lys154, Lys156, Arg159 and Arg161) that might be responsible for electrostatic interactions with the negatively charged phosphoinositides.

To test the involvement of these amino acids in the attachment of SteA to PI(4)P, we generated individual and combined mutations in these residues, and tested the localization of the different SteA versions *in vivo* in yeast cells by transforming *S. cerevisiae* with plasmids expressing GFP-SteA, GFP-SteA$^{3K\text{-}3A}$ (all three Lys26, Lys33 and Lys36 changed to Ala), GFP-SteA$^{2K2R\text{-}4A}$ (all four Lys154, Lys156, Arg159 and Arg161 changed to Ala), GFP-SteA^{K29A}, GFP-SteA^{K33A} or GFP-SteA^{K36A} (Fig. 30A). Protein expression was checked by immunoblotting, which showed that WT and mutant GFP-SteA proteins accumulated at similar levels (Fig. 31). As shown in figure 30A, changing all basic residues in the C-terminal cluster of SteA to alanines (GFP-SteA$^{2K2R\text{-}4A}$) did not affect localization of the protein, as this mutant version displayed a percentage of cells with PM-localized SteA similar to that of cells expressing WT SteA (Fig. 30B). In contrast, substitution of the three N-terminal basic residues led to complete loss of both PM and vacuolar localization (Fig. 30A and B), displaying instead a dotted pattern. All individual mutants GFP-SteA^{K29A}, GFP-SteA^{K33A} or GFP-SteA^{K36A} failed to localize to the PM (Fig. 30A and B). However, while GFP-SteA^{K29A}, GFP-SteA^{K33A} kept intact their vacuolar localization, GFP-SteA^{K36A} behaved like the triple mutant, showing a puncta-like pattern that appeared neither associated to the PM or to the vacuole (Fig. 30A). Therefore, Lys36 is essential for both PM and vacuolar localization of SteA in *S. cerevisiae*, while the two other neighboring lysine residues are only required for efficient interaction with the plasma membrane.

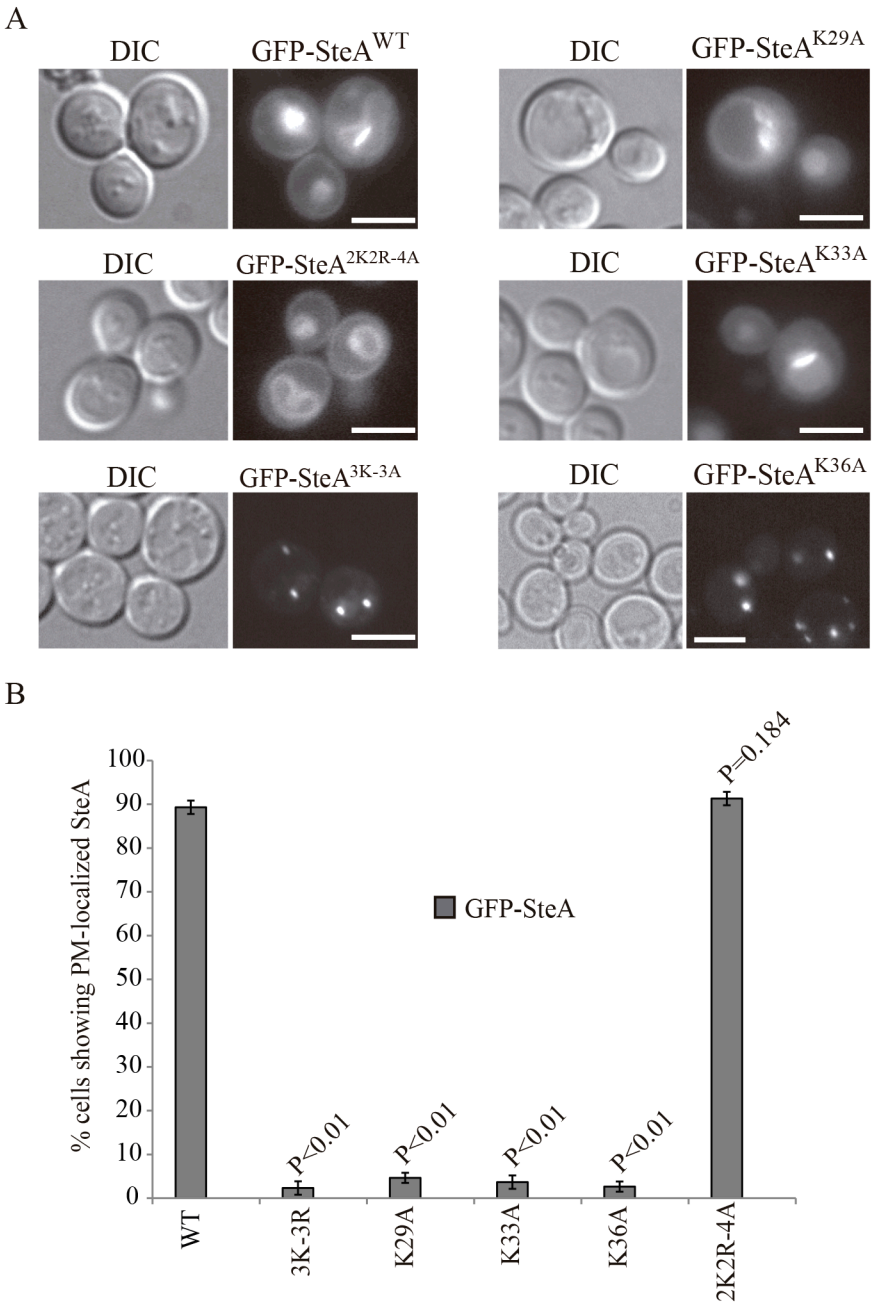

Figure 30. Effect of the mutation of basic residues on SteA localization in yeast cells.
(**A**) Differential Interference Contrast (DIC) and fluorescence images showing cell morphology (left panels) and localization (right panels) of GFP-SteA^{WT}, GFP-SteA$^{2K2R-4A}$, GFP-SteA^{3K-3A}, GFP-SteA^{K29A}, GFP-SteA^{K33A} and GFP-SteA^{K36A} expressed in *S. cerevisiae* YPH499 from pYES3GFP-based plasmids after 4 h incubation in SC-R (-Trp) media containing 2% galactose. All scale bars indicate 5µm. (**B**) Quantification of the percentage of cells showing PM-localized SteA. Bars in graphs indicate the standard deviation from three different clones; at least 100 cells were counted for each clone.

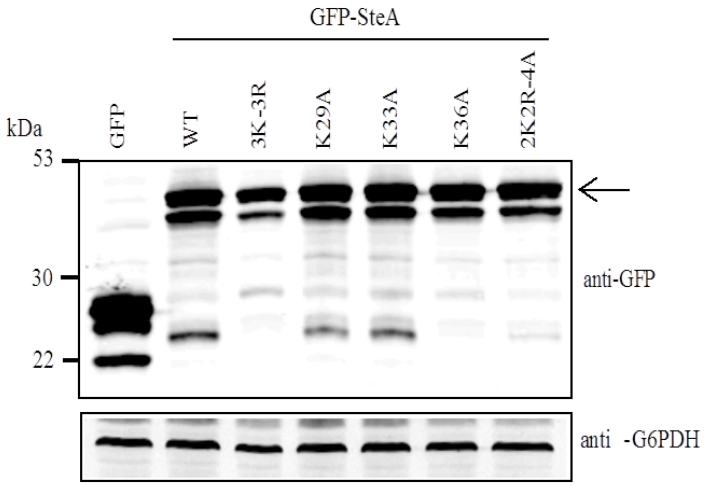

Figure 31. Expression of different SteA versions in yeast cells analysed by immunoblotting.
(A) Yeast YPH499 cells were transformed with pYES3GFP and pYES3GFP-SteA plasmids encoding wild type SteA or the mutant versions indicated. Yeast lysates obtained after 4 h of expression induction in SC-R (-Trp) media containing 2% galactose and analyzed by immunoblotting with anti-GFP and anti-glucose-6-phosphate dehydrogenase (G6PDH) antibodies (both used at 1:1000), and appropriate secondary antibodies. The arrows indicate the full length GFP-SteA bands.

II.9. The basic residue K36 of SteA is necessary to induce toxicity and mitochondrial condensation in *S. cerevisiae*

To check whether the basic residue K36 is essential or not for growth inhibitory effect in *S. cerevisiae,* yeast cells were transformed with plasmids expressing GST, GST-SteA or GST-SteA^{K36A}. As shown in figure 32, GST-SteA^{K36A} did not induce toxicity in yeast compared to GST and GST-SteA. Thus the residue K36 is necessary to induce toxicity of SteA when expressed in *S. cerevisiae.*

To test whether K36 was also required for the mitochondrial condensation caused by SteA, yeast cells were transformed with the plasmid expressing GST-SteA^{K36A} along with the mitochondrial marker Ilv6-DsCherry. As shown in the figure 33, no significant changes in mitochondrial morphology were observed in cells expressing the mutant version of SteA. These results support the idea that the toxicity of SteA in yeast is related with the condensation of mitochondria and confirm that the basic residue K36 is necessary both for proper SteA subcellular localization and for induction of this phenotype.

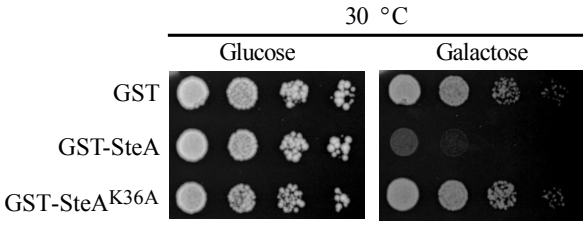

Figure 32. Effect of mutation on the lysine 36 of the GST-SteA on growth inhibition of *S. cerevisiae*. Ten-fold serial dilutions of cells (YPH499 strain) expressing either GST, GST-SteA or GST-SteA^{K36A} all from pEG(KG)-based plasmid, were spotted and incubated for 72 h in solid SC(-Ura-Leu) agar supplemented with glucose (repression conditions) or galactose (induction conditions) at 30°C.

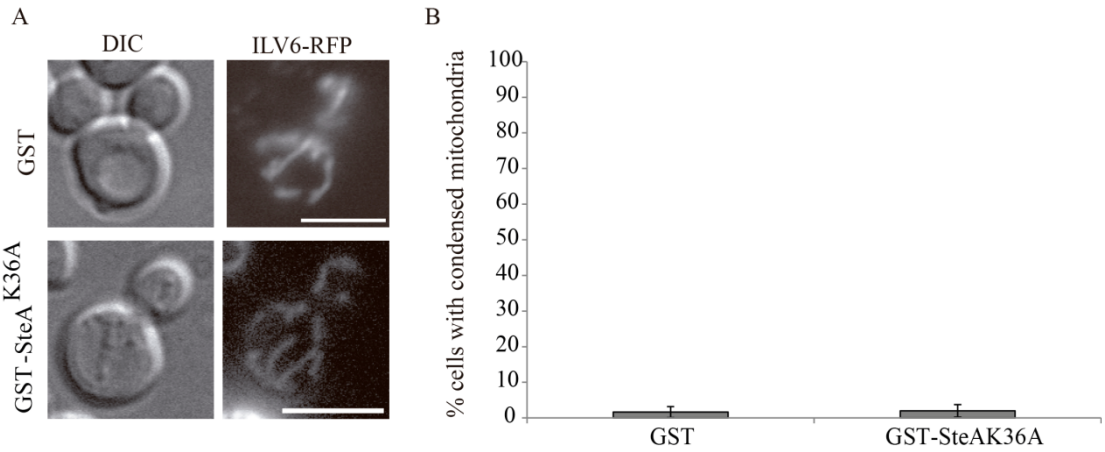

Figure 33. Mitochondrial morphology in yeast cells expressing GST-SteA^{K36A}
(A) Differential Interference Contrast (DIC) and fluorescence images showing cell morphology (left panel) and localization of the mitochondrial marker Ilv6-RFP (right panels) in *S. cerevisiae* YPH499 expressing GST or GST-SteA^{K36A} from pEG(KG)-based plasmids after 4 h incubation in SC-R (-Ura-Leu-Trp) media containing 2% galactose (w/v). All scale bars indicate 5µm. (**B**) Quantification of the percentage of cells with condensed mitochondria. Bars in graphs indicate the standard deviation from three different clones; at least 100 cells were counted for each clone.

II.10. Expression of SteA from the GFP fusion vectors does not induce toxicity and mitochondrial condensation in *S. cerevisiae*

Since GST-SteA induces toxicity when overexpressed in yeast, requiring double selection in order to force a higher number of copies of pEG(KG) plasmid (see I.2), we decided to check if GFP-SteA and SteA-GFP versions are toxic. Yeast cells expressing either GFP-SteA from episomic plasmids, pYES2GFP or pYES3GFP (*URA3*, and *TRP1* respectively), or SteA-GFP from centromeric YCpLG plasmid (*LEU2*) did not show a significant growth inhibition (Fig. 34). Thus, these results confirm the above

observation that a strong expression, such as that obtained with the pEG(KG) plasmid, is necessary to induce toxicity in yeast.

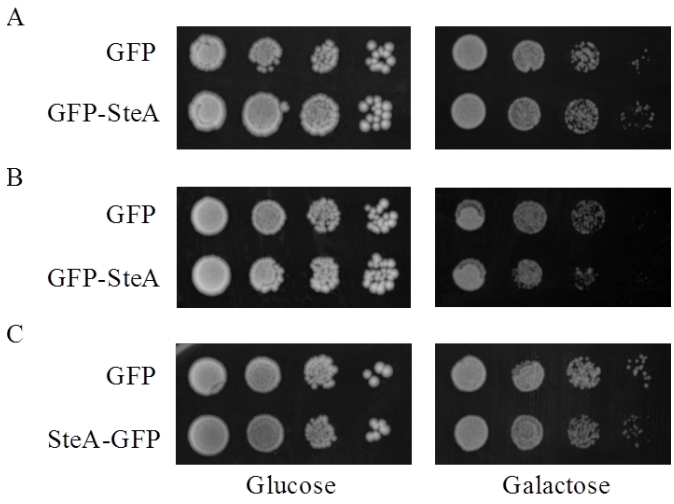

Figure 34. Effect of expression of full length GFP-SteA and SteA-GFP on *S. cerevisiae* growth. Ten-fold serial dilutions of yeast cells expressing (**A**) GFP and GFP-SteA (pYES3GFP-based plasmid), (**B**) GFP, and GFP-SteA (pYES2GFP-based plasmid), (**C**) GFP and SteA-GFP (YCpLG-GFP-based plasmid) expressed in *S. cerevisiae* YPH499, spotted and incubated for 72 h in solid (A) SC (-Trp), (B) SC(-Ura), (C) SC(-Leu) agar supplemented with glucose (repression conditions) or galactose (induction conditions) at 30°C.

III. STUDY OF SteA VACUOLAR LOCALIZATION in *S. cerevisiae*

III.1. Rab GTPase Ypt7 is not required for SteA localization in yeast

Ypt7 is the yeast homolog of the mammalian small GTPase Rab7, and is involved in the regulation of endocytic traffic from late endosome/MVB to the vacuole, being required for homotypic vacuole fusion (Schimmöller & Riezman., 1993). *ypt7* null mutants have been shown to display small fragmented vacuoles (Kashiwazaki, *et al*., 2009). Ypt7 interacts as well with the cargo selection/retromer complex for retrograde sorting from endosomes to Golgi (Liu *et al*., 2012). Some bacterial effectors manipulate the activity of Rab GTPases thus favoring the survival of the intracellular pathogen (Ham *et al*., 2011). Therefore, we wanted to check whether SteA requires Ypt7 in order to be localized to the yeast vacuole. Wild type yeast and *ypt7*Δ strains were transformed with plasmid expressing GFP-SteA. As shown in figure 35B SteA localized at the PM and also at the abnormal vacuoles displayed by *ypt7*Δ mutant cells. Therefore, the Ypt7 Rab is dispensable for SteA targeting to the vacuole.

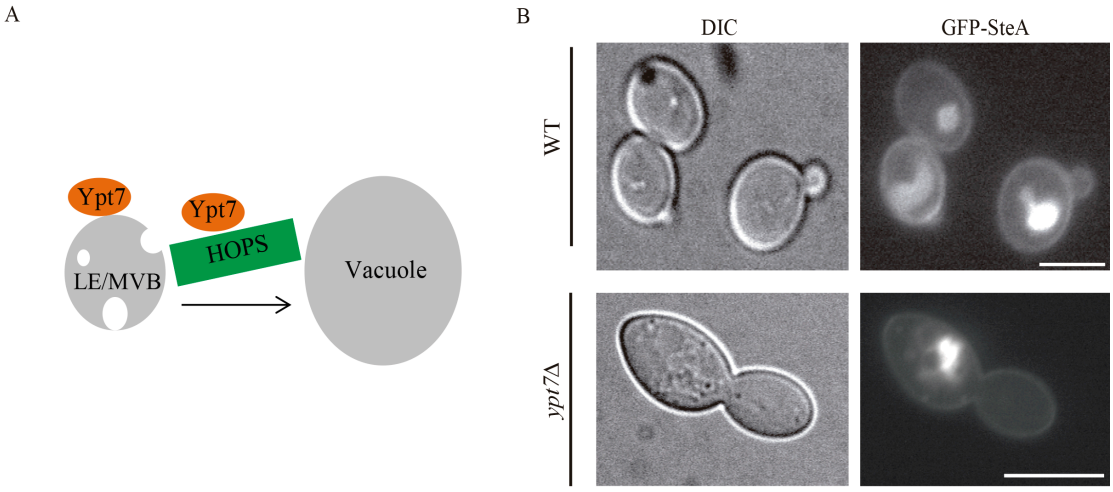

Figure 35. Localization of SteA in a *ypt7* mutant, defective at sorting at the MVB level.
(A) Schematic representation of protein Ypt7 implicated in sorting at MVB and homotypic vacuole fusion. (**B**) Differential Interference Contrast (DIC) and fluorescence images showing cell morphology (left panel) and localization of GFP-SteA (right panel) expressed in *S. cerevisiae* BY4741 (WT) and Y00575 (BY4741; *ypt7::kanMX4*) from plasmid pYES2GFP-SteA, after 4 h incubation in SC-R (-Ura) media containing 2% galactose (w/v).

III.2. Mutation in Class A *Vps* genes do not affect SteA vacuolar localization, whereas in Class C vps mutants SteA is retained in pre-vacuolar endosomes

To know whether recruitment of SteA to the vacuole requires specific trafficking pathways we studied localization of GFP-SteA in a set of vacuolar protein sorting mutants (*vps*) affected in different stages of vesicle transport towards the vacuole (Bowers & Stevens, 2005).

In wild type cells, the precursor p2 form of the carboxypeptidase Y (CPY) is transported to the vacuole where it is processed to mature mCPY form (Fig. 36A). However, *vps* mutants mis-sort a portion of p2CPY to the secretory pathway (Bower & Stevens, 2005). Pep1/Vps10 is the CPY receptor that controls the trafficking of CPY out of the late Golgi to the late endosome or MVB, also known as pre-vacuolar compartment (Fig. 36A) (Marcusson *et al*., 1994). The *vps10* mutant belongs to the class A *vps* mutants that display a wild type vacuolar morphology. In order to check if the function of Vps10 was essential to target SteA towards the vacuole, we transformed the wild type and an isogenic strain harboring a deletion in the *VPS10* gene with a plasmid expressing GFP-SteA. As shown in figure 36C, PM and vacuolar GFP-SteA signals were not significantly affected by the mutation of the typical class A *pep1/vps10* mutant.

Class C Vps act at two distinct protein trafficking steps: fusion of late Golgi vesicles with late endosomes/MVB, and fusion of the latter to the vacuole (Bowers & Stevens, 2005). Therefore, class C mutants accumulate fusion-incompetent endosomes and vesicles in the cytoplasm of the cell and lack identifiable vacuoles. SteA localized at the PM and accumulated in putative abnormal endosomes in class C *vps16* mutant (Fig. 36D).

Thus these results suggest that Class A Vps proteins are not required for SteA targeting to the vacuole whereas the lack of Class C Vps proteins does not affect SteA trafficking through the endocytic pathway but impedes SteA vacuolar localization due to the absence of distinguishable vacuoles.

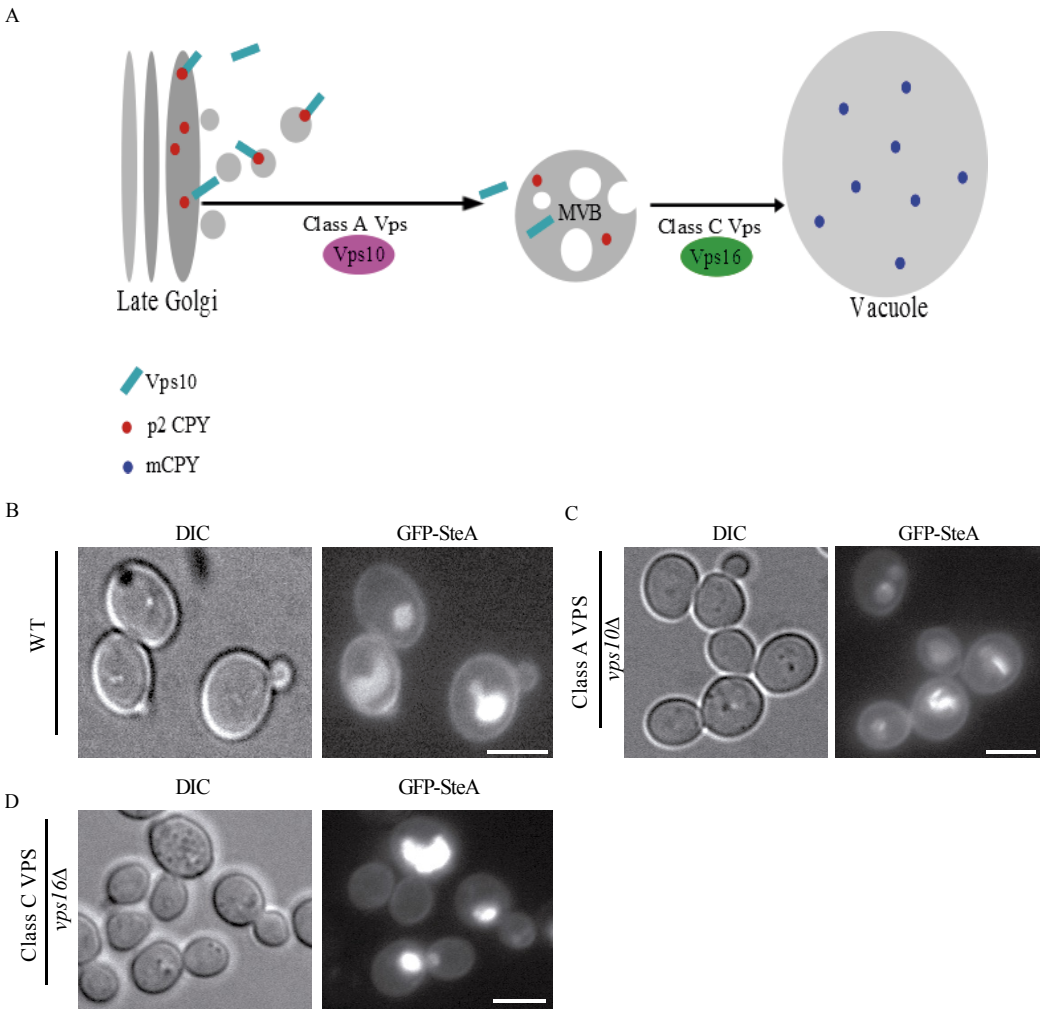

Figure 36. Localization of SteA in Golgi-to-vacuole traffic mutants
(A) Schematic presentation of vacuole protein sorting (Vps) proteins implicated in different stages of the endosomal trafficking; p2CPY binds to Vps10 and the complex is sorted towards late endosome, where it is cleaved to generate mature CPY (mCPY). (**B**) Differential Interference Contrast (DIC) and fluorescence images showing cell morphology (left panel) and localization of GFP-SteA (right panel) expressed in *S. cerevisiae* BY4741 (WT) from plasmid pYES2GFP-SteA, after 4 h incubation in SC-R (-Ura) media containing 2% galactose (w/v). (**C**) *S. cerevisiae* Y05072 (BY4741; *vps10::kanMX4*) expressing GFP-SteA processed and analyzed as in B. (**D**) *S. cerevisiae* Y02783 (BY4741; *vps16::kanMX4*) expressing GFP-SteA processed and analyzed as in B. All scale bars indicate 5 µm.

III.3. SteA requires class D Vps proteins to localize at the vacuole

We next checked mutants affected in class D Vps function. These proteins are required for fusion of vesicles with the late endosome/MVB and their absence leads to the presence of a single large vacuole that fails to extend into daughter cells (Bowers &

Stevens, 2005). To analyze the effect of the lack of some of those class D Vps proteins, wild type yeast and isogenic strains harboring *vps3*Δ, *vps8*Δ or *vps45*Δ mutations were transformed with plasmid expressing GFP-SteA. The *vps3* and *vps8* mutant cells did not lose SteA PM localization. In contrast to the WT cells, they did not show SteA in the vacuole but it seemed to accumulate in large puncta adjacent to the vacuole. In *vps45* mutant cells SteA accumulated in small dots throughout the cytoplasm adjacent or not to the vacuole, as well as, peculiary, losing PM localization (Fig. 37C). These results suggest that Class D proteins are required for targeting GFP-SteA to the vacuole.

In order to identify the nature of the dots where SteA accumulated in class D mutants, we stained both *vps8*Δ and *vps45*Δ mutants transformed with GFP-SteA with the endosomal FM4-64 fluorescent marker. As shown in figure 39, the GFP-SteA signal in these compartments did not co-localize with FM4-64, csuggesting that they are not late endosomes.

III.4. Protein sorting at the multivesicular body involving Class E Vps proteins is essential for the traffic of SteA to the vacuole

In order to investigate further the involvement of other elements of the endocytic traffic in targeting SteA to the yeast vacuole, we decided to work with class E *vps* mutants (Fig. 38A). Class E Vps proteins are involved in the sorting of proteins at the MVB for delivery to the vacuole (Bowers & Stevens, 2005). The class E *vps* mutants displayed a large and aberrant endosome/MVB (the class E compartment) adjacent to the vacuole that contains vacuolar hydrolases and vacuolar membrane proteins, and are defective in protein recycling to the late Golgi.

Wild type and some class E *vps* mutant strains from the EUROSCARF deletion collection were transformed with GFP-SteA. In contrast to WT cells, in *vps27*Δ, *vps23*Δ, *vps22*Δ and *vps20*Δ corresponding to ESCRT 0, ESCRT I, ESCRT II, and ESCRT III complexes, respectively, GFP-SteA localized faintly at the vacuolar membrane and accumulated in a compartment adjacent to the vacuole, but not in the vacuolar lumen (Fig. 38B). These data suggest that in class E mutants, SteA was not able to be sorted into luminal MVB vesicles, and was retained in the class E

compartment. Thus, SteA is targeted to the yeast vacuolar lumen by ESCRT-mediated sorting at the MVB.

In order to confirm that the GFP-SteA-marked compartments that appear in class E mutants are late endosomes/MVB, we took two class E mutant *vps* (*vps20*Δ, and *vps23*Δ) transformed with GFP-SteA and stained endosomal membranes with the FM4-64 fluorescent marker. As shown in figure 39, unlike in class D mutants GFP-SteA cytoplasmic spots co-localized with FM4-64 in class E mutants, confirming that the compartment adjacent to the vacuole where SteA accumulates in these mutants is indeed the class E compartment.

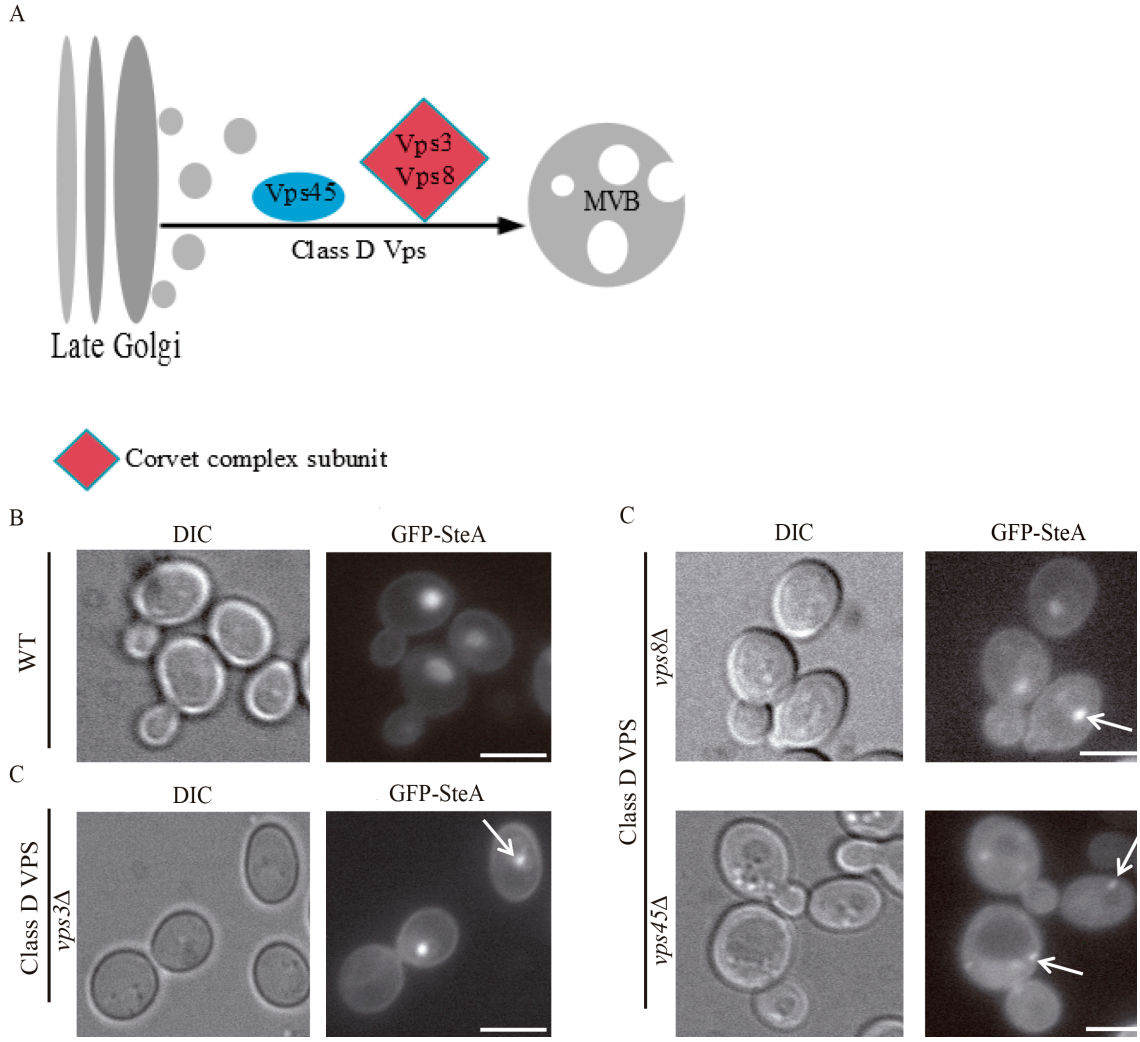

Figure 37. Alteration of SteA vacuolar localization in Class D *vps* mutants

(A) Schematic presentation of the indicated Vps proteins in different stages of endosomal trafficking. **(B)** DIC and fluorescence microscopy images showing cell morphology (left panels) and localization of GFP-SteA (right panels) in *S. cerevisiae* BY4741 (WT) expressing GFP-SteA from pYES2GFP-SteA, after 4 h incubation in SC-R (-Ura) media containing 2% galactose (w/v).**(C)** *S. cerevisiae* Y04329 (BY4741; *vps3::kanMX4*), Y00405 (BY4741; *vps8::kanMX4)* and Y04462 (BY4741; *vps45::kanMX4)* expressing GFP-SteA from pYES2GFP-SteA were processed and analyzed as in B. All scale bars indicate 5 µm.

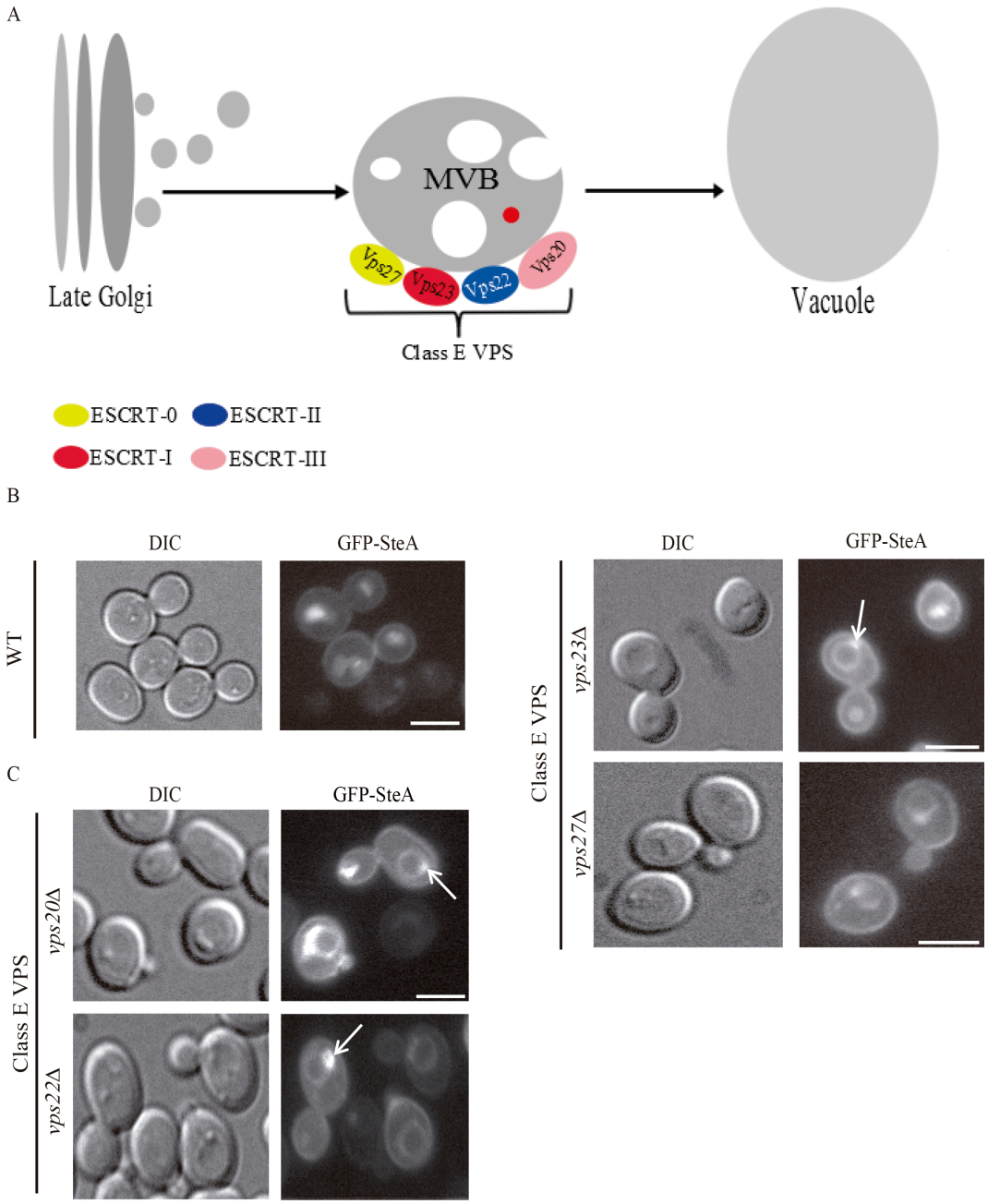

Figure 38. Alteration of SteA vacuolar localization in Class E *vps* mutants
(A) Schematic presentation of the indicated Vps proteins implicated in different stages of endosomal trafficking. (**B**) DIC and fluorescence microscopy images showing cell morphology (left panels) and localization of GFP-SteA (right panels) in *S. cerevisiae* BY4741 (WT) transformed with pYES2GFP-SteA, after 4 hs incubation in SC-R (-Ura) media containing 2% galactose (w/v). (**C**) Transformants of the BY4741 strain derivatives deleted in indicated Class E *VPS* genes were processed and analyzed as in **B**. All scale bars indicate 5 µm.

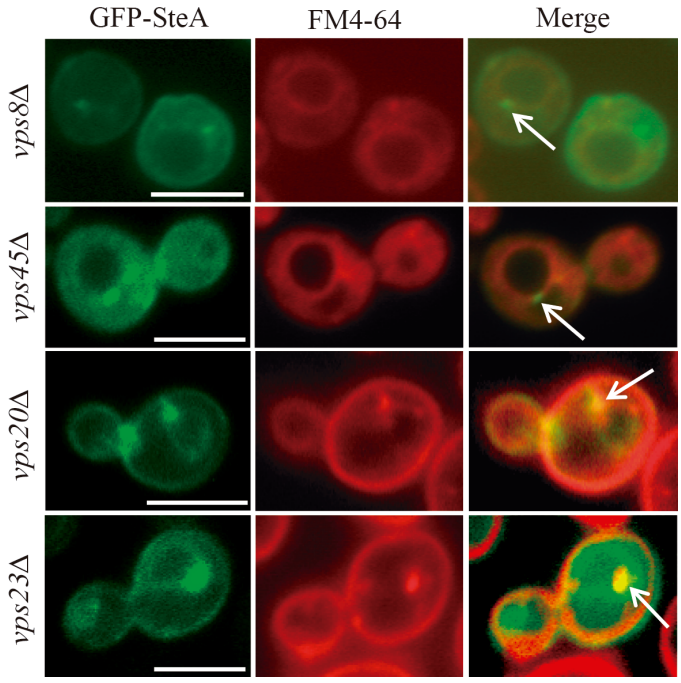

Figure 39. Co-localization analysis of GFP-SteA with the endocytic marker FM4-64 in class D and E *vps* mutants. Fluorescence microscopy images showing GFP-SteA in *S. cerevisiae* BY4741 derivative strains Y00405, Y04462, Y06211 and Y03416 (*vps8::kanMX4, vps45::kanMX4, vps20::kanMX4* and *vps23::kanMX4,* respectively) expressing GFP-SteA from pYES2GFP-SteA plasmid after induction in SC-R(-Ura) supplemented with 2% galactose (w/v) for 4 hours and treated with the endocytic marker FM4-64 for 1 h. All scale bars indicate 5 µm.

III.5. Endosome to Golgi retrograde transport is not required for SteA targeting to the yeast vacuole

Since we had checked the effect of the mutation of different classes of *vps* mutants, we decided to check the GFP-SteA localization also in class F mutants, like *vps26*Δ, which possess one large vacuole often surrounded by fragmented vacuolar structures (Bowers & Stevens, 2005). Vps6 is one the proteins of the retromer complex and, therefore, mutant cells lacking this protein are defective in the endosome to Golgi retrograde protein transport. We did not notice any difference in the vacuolar localization of SteA in the *vps26*Δ mutant (Fig. 40C). Thus, a functional retromer is not required for proper SteA localization to the yeast vacuole.

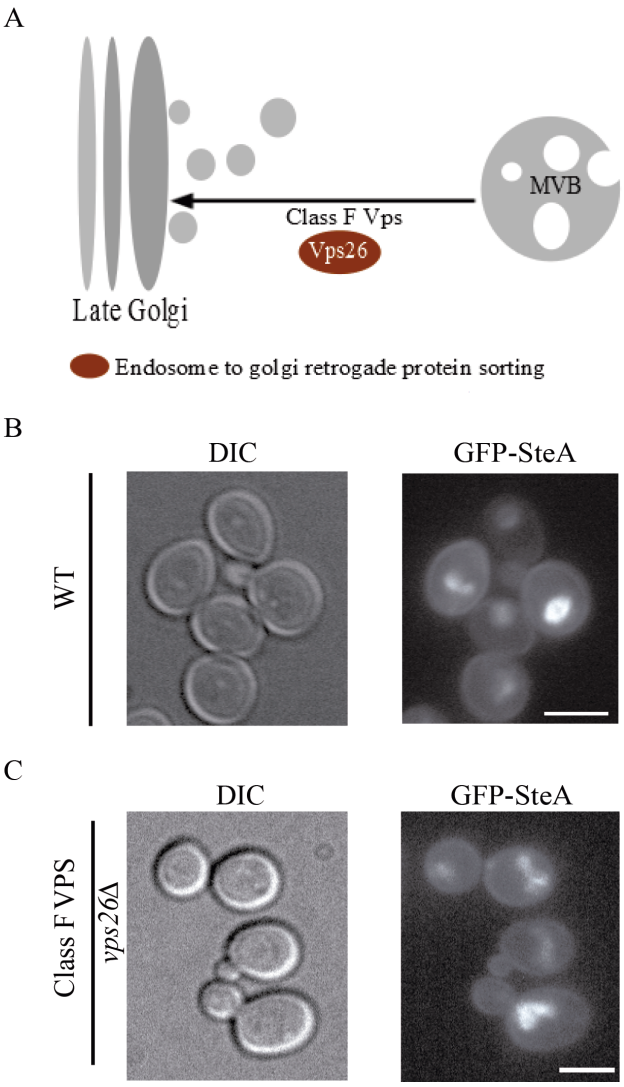

Figure 40. Localization of SteA in Golgi-to-vacuole Class F *vps* mutants
(**A**) Schematic representation of Vps26 implication in endosomal to Golgi retrograde trafficking. **(B**) DIC and fluorescence microscopy images showing cell morphology (left panels) and localization of GFP-SteA (right panels) in *S. cerevisiae* BY4741 (WT) expressing GFP-SteA from pYES2GFP-SteA, after 4 h incubation in SC-R (-Ura) media containing 2% galactose (w/v). (**C**). *S. cerevisiae* derivative from BY4741, Y01370 *(vps26::kanMX4)* expressing GFP-SteA from plasmid pYES2GFP-SteA was processed and analyzed as in **B.** All scale bars indicate 5 µm.

IV. Search for interactors of SteA in *S. cerevisiae*

IV.1. Components of the Fab1 complex interact genetically with SteA

The *S. cerevisiae* Fab1 phosphoinositide phosphate kinase is a conserved large protein, homologous to mammalian PIKfyve, associated with membrane PI(3)P and involved in PI(3,5)P_2 biosynthesis (Shisheva, 2008; Rusten *et al*., 2006). A mutant lacking Fab1 function is affected in later stages of the endocytic pathway, displaying defects in retrograde trafficking from the vacuole to the Golgi and trafficking of some proteins into the vacuole via MVB (Dove *et al*., 2002). Fab1 binds to Vac14 and the phosphoinositide 5-phosphatase Fig4 and forms a vacuole-associated signaling complex (Botelho *et al*., 2008). Interestingly, in a work with our collaborator John Brumell based on proteomic experiments using mammalian cell extracts (HEK293T expressing SteA), we found a possible interaction of SteA with Vac14, the scaffold protein of the PIKfyve complex. So we wanted to test the effect of the absence of the components of the Fab1 complex on the effect of SteA in *S. cerevisiae* cell growth. Wild type BY4741 and isogenic *vac14*Δ, *fab1*Δ, and *fig4*Δ mutant strains were transformed with plasmids expressing either GST or GST-SteA, and spot growth assays on agar were performed. As shown in figure 41B, SteA overexpression caused a stronger growth inhibition in cells harboring either *vac14*Δ or *fab1*Δ, but not *fig4*Δ, than in the wild type strain. Thus, these results denote a phenotypic enhancement of the toxic effect of SteA as a consequence of the lack of Vac14 and Fab1.

IV.2. SteA does not interact physically with Vac14

Genetic interactions not always reflect a direct physical interaction of the proteins encoded. However, in the knowledge that SteA may physically bind to the Vac14-Fab1 complex in higher cells (Brumell Lab, unpublished results), we wanted to check whether SteA physically interacted with Vac14 in yeast. We performed a co-purification experiment in a glutathione matrix with cellular extracts expressing GST or GST-SteA and HA-tagged Vac14 and then pulled down the proteins using antibody anti-GST. Then Western blots were developed using anti-GST and anti-HA antibodies. As observed in figure 42, we did not see any specific physical interaction between SteA and

Vac14 by this approach, since GST-SteA pulled down Vac14-HA to the same extent as GST alone did.

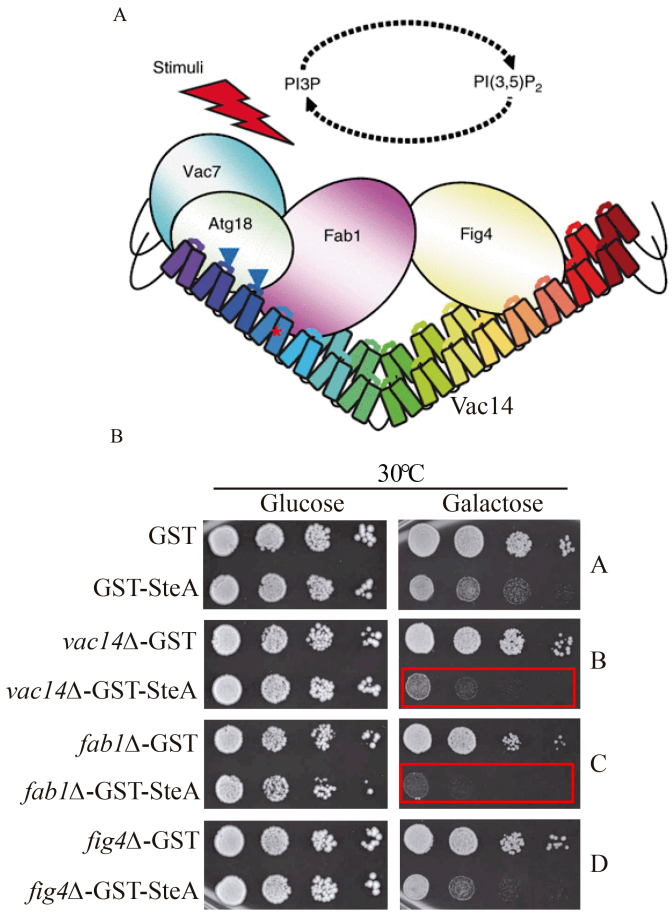

Figure 41. Effect of deletion of the Fab1 complex subunits on GST-SteA-induced growth inhibition of *S. cerevisiae*. (A) Schematic representation of the Fab1 complex. **(B)** Ten-fold serial dilutions of BY4741 (WT) and the indicated isogenic mutants cells, expressing either GST or full length GST-SteA from pEG(KG) or pEG(KG)-SteA respectively, were spotted and incubated for 72 h in solid SC(-Ura-Leu) agar supplemented with glucose (repression conditions) or galactose (induction conditions) at 30°C.

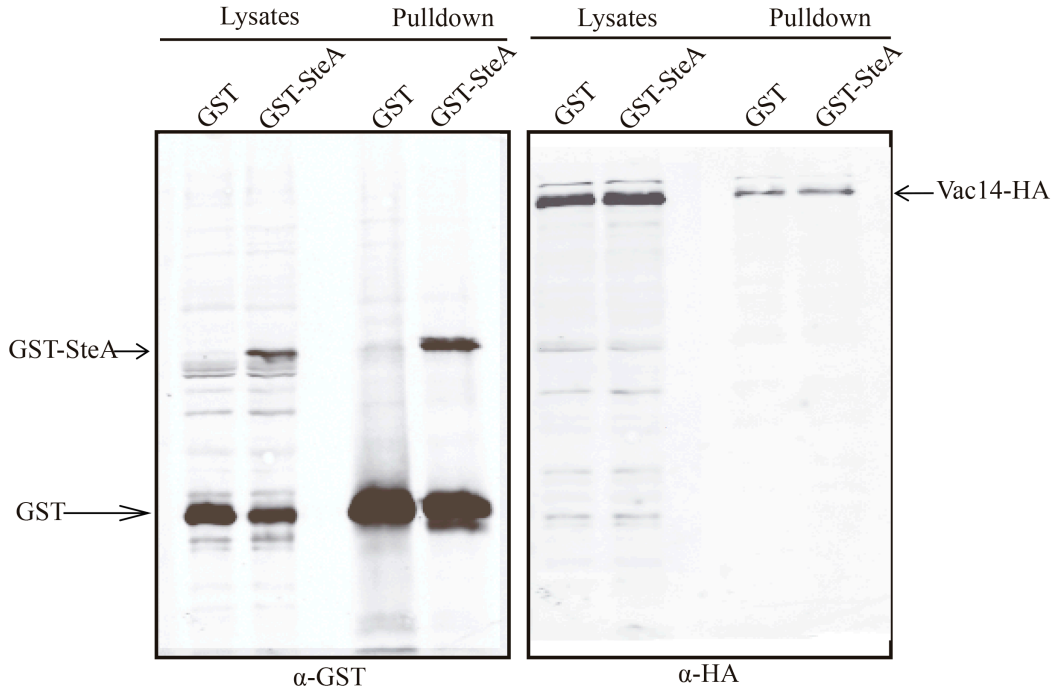

Figure 42. Co-purification assay of SteA and Vac14.
Western blotting analysis of proteins extracts (lysates) and proteins retained (pulldown) in a co-purification experiment using a glutathione matrix (*Glutathione Sepharose*). Yeast strain RBY64 (*VAC14-HA::TRP1*) was transformed with plasmid pEG(KG) or pEG(KG)-SteA expressing GST or GST-SteA respectively. Cells were grown in SC-R (-Ura-Trp) supplemented with galactose 2% (w/v) for 4 hours. The antibodies used were anti-HA (right panel) in order to detect Vac14-HA, and anti-GST (left panel) to analyze the presence of GST and GST-SteA.

IV.3. SteA targeting to the vacuole does not requires Fab1 complex components

After studying the effect of the mutation of the components of the Fab1 complex, we decided to check whether the Fab1 complex affects SteA trafficking towards the vacuole. To this end, wild type *S. cerevisiae* and isogenic strains harboring mutations in components of the Fab1 complex (*vac14*Δ, *fab1*Δ, and *fig4*Δ) were transformed with a plasmid expressing GFP-SteA. None of the mutants showed any changes in GFP-SteA signal at the vacuole (Fig. 43). Therefore trafficking of SteA towards the vacuole does not require the Fab1 complex.

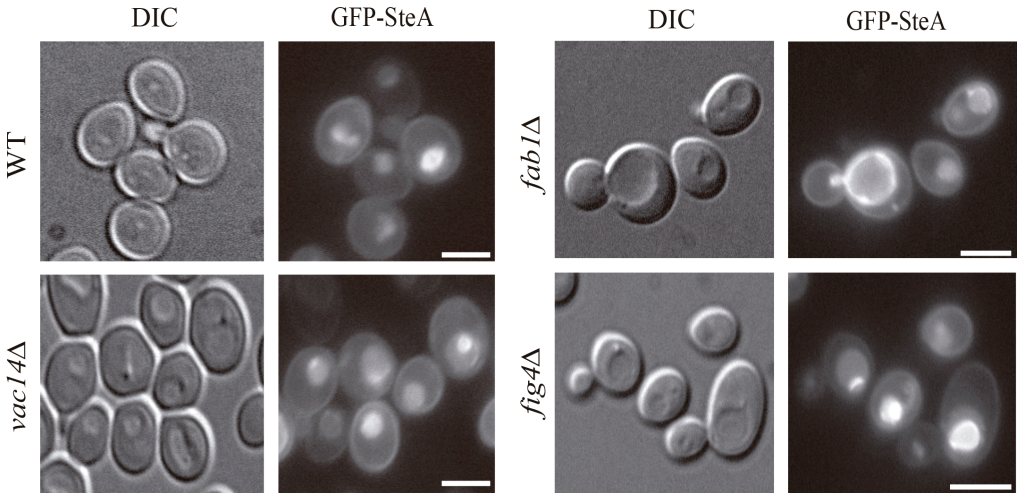

Figure 43. Localization of GFP-SteA in Fab1 complex mutant yeast cells.
DIC and fluorescence microscopy images showing cell morphology (left panels) and localization of GFP-SteA (right panels) in *S. cerevisiae* BY4741 (WT) or the indicated isogenic mutant strains [Y05295 (*vac14::kanMX4*), Y07080 (*fab1::kanMX4*) and Y01120 (*fig4::kanMX4*)], expressing GFP-SteA from plasmid pYES2GFP-SteA, after 4 h of incubation in SC-R (-Ura) media containing 2% galactose (w/v). All scale bars indicate 5 µm.

IV.4. Search for possible SteA interactors among known negative genetic interactors of *vac14* and *fab1* mutants

In order to detect cellular functions that may account for SteA genetic interaction with *vac14* and *fab1*, we looked into the list of negative genetic interactors with these mutants available at SGD and selected those common to both of them. We came up with eleven possible candidates genes that, when deleted, had been reported to negatively affect growth of *vac14* and *fab1* mutants (Table IX). First, we checked the growth inhibitory effect of SteA overexpression in cells harboring mutations in each of these eleven genes. As shown in figure 44, four (marked in red) of the selected mutants (lacking Cla4, Vps3, Vps35 or Vps1), showed a phenotypic enhancement of SteA-induced toxicity, indicating a negative genetic interaction with SteA overexpression. Interestingly, 3 out of these 4 mutants are involved in vacuolar protein sorting in yeast (*vps1*Δ, *vps3*Δ, and *vps35*Δ).

We checked the localization of GFP-SteA in these mutants. As already shown in Fig. 37, class D mutant *vps3*Δ displayed an altered GFP-SteA localization. However, neither in class A *vps35*Δ nor in class F *vps1*Δ mutant cells, the vacuolar localization of

SteA was affected (Fig. 45). No changes in SteA localization were observed in mutant cells lacking the protein kinase Cla4 (Fig. 45).

Table IX. List of the 11 genes described to display a negative genetic interaction with both *fab1* and *vac14*, and their cellular function in *S. cerevisiae* according to the yeast genome database (SGD).

Gene/ORF	Description
***ATG18* / YFR021W**	-Phosphoinositide binding protein -Vesicle formation in autophagy and the cytoplasm-to-vacuole targeting (Cvt) pathway
***CLA4* / YNL298W**	-Cdc42p-activated signal transducing kinase -Involved in septin ring assembly, vacuole inheritance, cytokinesis, sterol uptake regulation
***PEP8* / YJL053W**	-Endosome-to-Golgi retrograde protein transport
***BEM2* / YER155C**	-Rho GTPase activating protein -Control of cytoskeleton organization, cellular morphogenesis, ad bud emergence
***VPS3* / YDR495C**	-Component of CORVET complex -Required for the sorting and processing of soluble vacuolar proteins -Assembly of the V-ATPase -Acidification of the vacuolar lumen
***VPS29* / YHR012W**	-Endosomal protein -Endosome-to-Golgi retrograde protein transport
***VPS30* / YPL120W**	-Subunit of phosphatidylinositol (PtdIns) 3-kinase complexes I (autophagy) and II (vacuolar protein sorting)
***VPS35* / YJL154C**	-Endosomal subunit of membrane-associated retromer complex
***VPS1* / YKR001C**	-Dynamin-like GTPase -Actin cytoskeleton organization -Late Golgi retention of some proteins
***YPT35* / YHR105W**	-Unknown function -Contains a phox (PX) homology domain -Binds to PI(3)P, and proteins involved in ER-Golgi transport
***VPS38* / YLR360W**	-Functions in carboxypeptidase Y (CPY) sorting -Binds Vps34 and Vps30 to promote production of PI(3)P

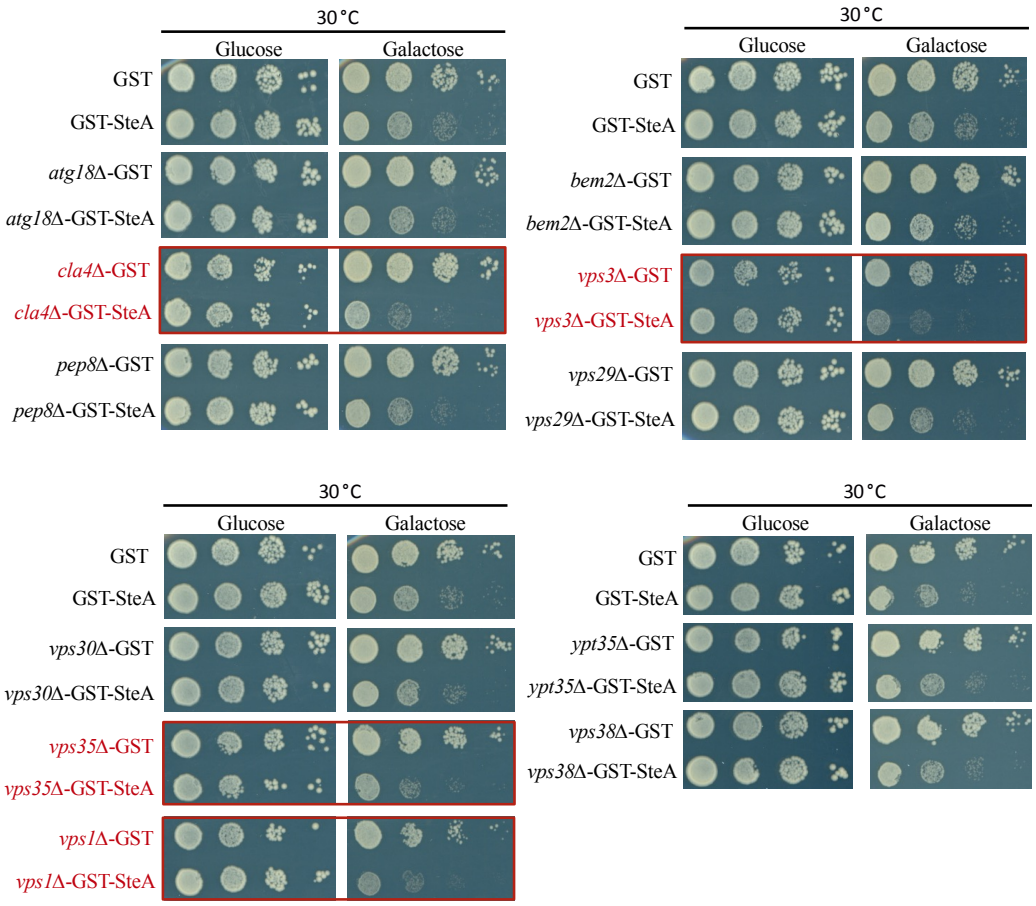

Figure 44. Effect of the absence of common *vac14* and *fab1* negative genetic interactors on SteA-induced growth inhibition in *S. cerevisiae.*

Ten-fold serial dilutions of wild type BY4741 (WT) and the indicated isogenic mutant strains transformed with plasmid pEG(KG) or pEG(KG)SteA expressing GST or GST-SteA respectively. Cells were spotted and incubated for 72 h in solid SC (-Ura-Leu) agar supplemented with glucose (repression conditions) or galactose (induction conditions) at 30℃.

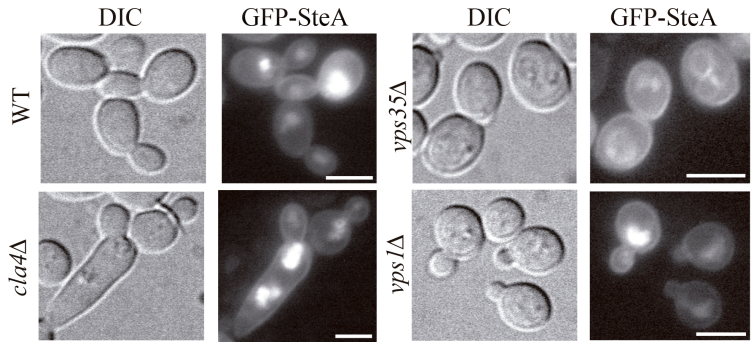

Figure 45. Localization of GFP-SteA in mutant strains showing phenotypic enhancement of SteA-induced toxicity. DIC microscopy showing cell morphology (left panels) and fluorescence microscopy showing localization of GFP-SteA (right panels) in *S. cerevisiae* BY4741 (WT) and its derivatives deleted in the indicated genes, expressing GFP-SteA from plasmid pYES2GFP-SteA, after 4 h incubation in SC-R (-Ura) media containing 2% galactose (w/v). All scale bars indicate 5µm.

IV.5. Voa1 is a phenotypic suppressor of SteA-induced growth impairment in yeast

Based on the results above described (IV.1 and IV.4) and following a similar rationale, we wanted to check the effect of overexpressing SteA and its localization in mutants reported to show common negative genetic interaction with mutants lacking Vps35, Cla4, Vps1 or Vps3. We found in the SGD database that mutants deleted in *VOA1*, *VMA21*, *VMA1*, and in the gene encoding the dynein light intermediate chain Dyn3 are among those interactors. These were interesting putative SteA targets because the vacuolar ATPase complex might be a binding-target for SteA that could explain its vacuolar localization, and because a dynein defect might account for the abnormal mitochondrial morphology observed. In *S. cerevisiae* the vacuolar ATPase (V-ATPase) complex is divided into two main sectors, the V_0 and V_1. V_0 is responsible of translocating protons, resulting in acidification of the organelle, and the V_1 catalyzes ATP hydrolysis. Voa1 and Vma21 are involved in the assembly of V_0 in the ER, and Vma1 is one of the subunits of the V_1 domain (Ryan *et al*., 2008).

We first tested the effect of deleting these genes on SteA-induced growth inhibition by performing serial dilution spot assays in wild type *S. cerevisiae* and the corresponding isogenic mutant strains transformed with plasmids expressing either GST or GST-SteA. As shown in figure 46, none of the mutants led to significant phenotypic enhancement of SteA overexpression but instead cells harboring *voa1*Δ showed phenotypic suppression. Thus, SteA might be targeting Voa1 in *S. cerevisiae*.

IV.6. SteA does not interact with Voa1 in *S. cerevisiae*

Since Voa1 showed a phenotypic suppression of SteA-induced growth effect, we wanted to check whether SteA interact physically with Voa1. To do this, we performed a co-immunoprecipitation experiment using antibodies anti-HA to immunoprecipitate HA-Voa1 in cells co-expressing GFP-SteA. As shown in figure 47, we did not find a specific interaction of HA-Voa1 with GFP-SteA over non-specific binding of HA-Voa1 to the GFP alone control.

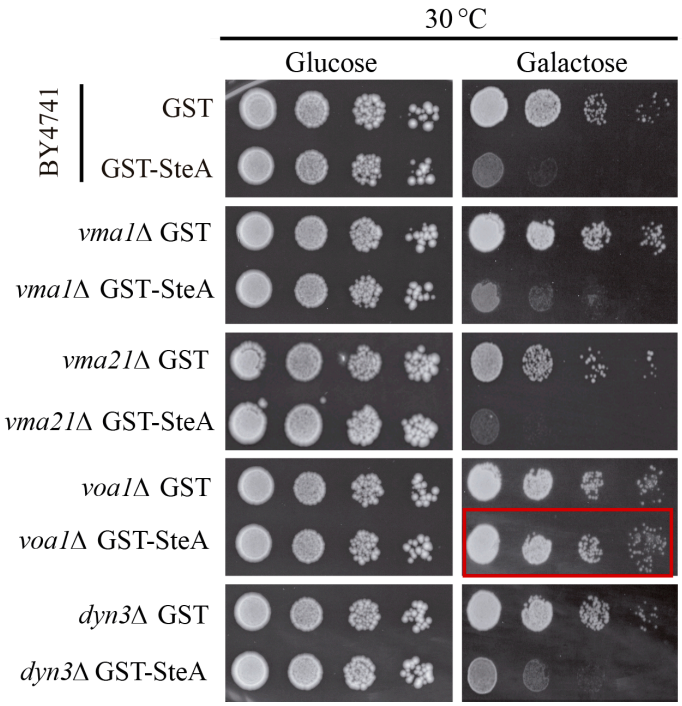

Figure 46. Effect of deletion of genes encoding proteins of the vacuolar ATPase complex and the dynein light intermediate chain on SteA-induced growth inhibition of *S. cerevisiae*.
Ten-fold serial dilutions of wild type BY4741 (WT) and the indicated isogenic mutant strains expressing either GST or GST-SteA from plasmid pEG(KG) or pEG(KG)SteA, respectively, were spotted and incubated for 72 h in solid SC(-Ura-Leu) agar supplemented with glucose (repression conditions) or galactose (activation conditions) at 30℃.

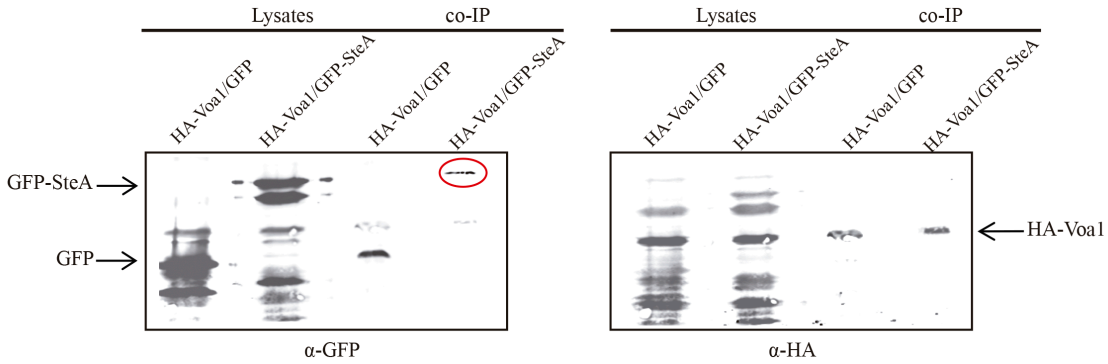

Figure 47. Co-purification assay of SteA and Voa1.
Western blotting analysis of protein lysates and proteins retained (co-IP) by co-immunoprecipitation assay. Yeast YPH499 cells expressing both GFP (pYES3-GFP) and HA-Voa1 (pMR072-HA-Voa1), or GFP-SteA (pYES3-GFP-SteA) and HA-Voa1 were grown in SC-R (-Ura-Trp) and galactose 2% (w/v) for 4 h and immunoblotted. The antibodies used were anti-HA (right panel) in order to detect HA-Voa1 , and anti-GFP (left panel) to analyze the presence of GFP and GFP-SteA.

IV.7. SteA vacuolar localization is not altered in *vma1*Δ, *vma21*Δ, *voa1*Δ and *dyn3*Δ *S. cerevisiae* mutants

We also checked if the vacuolar localization of SteA was affected in these mutants but, as shown in figure 48, none of the cells showed significant changes in SteA vacuolar localization compared to wild type cells. So Vma1, Vma21, Voa1, and Dyn3 are not involved in targeting SteA to the vacuole in yeast.

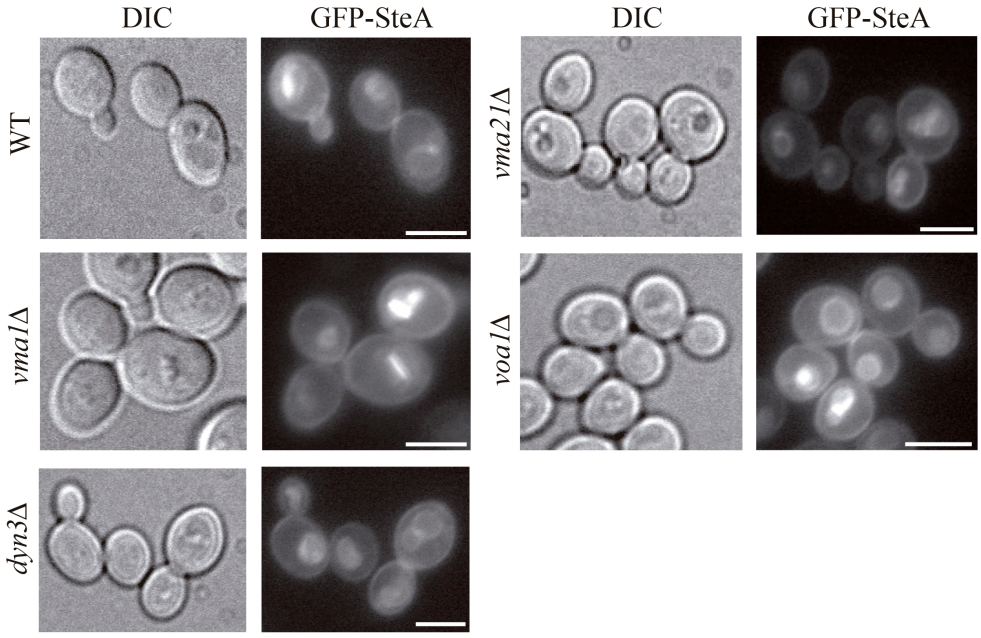

Figure 48. SteA vacuolar localization is not altered in deletion mutants related to vacuolar ATPase or dynein.
Differential Interference Contrast (DIC) and fluorescence images showing cell morphology (left panel) and localization of GFP-SteA (right panel) in *S. cerevisiae* BY4741 (WT) or the indicated isogenic mutant strains transformed with the plasmid expressing GFP-SteA after 4 h incubation in SC-R (-Ura) media containing 2% galactose (w/v). All scale bars indicate 5µm.

DISCUSSION

I. SteA: A *Salmonella* T3SS effector protein that triggers mitochondrial condensation in *S. cerevisiae*

Salmonella is an intracellular facultative pathogen found in the gastrointestinal tract of avian, mammalian and reptilian hosts (Malik-Kale *et al*., 2011) that, like other pathogenic enterobacteria, has developed a complex system of effector proteins that are injected into the cytoplasm of the host cell by specialized secretion systems. Once inside the cell, these effector proteins are able to manipulate diverse biochemical mechanisms and cellular structures with two main objectives: to allow the entrance of the bacterium across the plasma membrane of the host intestinal epithelium cells and to assure its intracellular survival inside the SCV, avoiding the activation of the immune defense mechanisms (Garmendia *et al.*, 2003; Kuhle & Hensel, 2004). In order to do this, *Salmonella* relies on T3SSs (encoded by SPI-1 and SPI-2) secreting two sets of bacterial effectors. Each effector protein is secreted at the opportune moment and acquires the most appropriate intracellular localization. In this way, all these effectors act in a concerted way, exerting a great variety of effects on different cellular biochemical pathways (Figueira & Holden, 2012; LaRock *et al.*, 2015). In addition, certain effectors may have more than one biochemical activity, which may be manifested at the same time or at different times of infection (Dean, 2011). Among *Salmonella* effectors, there are some proteins whose elimination does not cause a significant loss of virulence due to the existence of other effectors that perform a similar function, so that the effects of their individual elimination are masked and only small variations occur in the phenotype of the infected cell, which may be difficult to detect. A good example of this is the case of SopE, SopE2 and SopB that redundantly mediate the positive regulation of Rho GTPases required in the directed phagocytosis process, so that only in a triple mutant *sopEΔsopE2ΔsopBΔ* the ability of *Salmonella* to invade non-phagocytic cells is completely inhibited (Molero *et al.*, 2009; Ramos-Morales, 2012). However, there are other effector proteins whose function is crucial for the infection process, to the point that their lack results in a total or partial loss of the virulence of the bacterium, which can manifest itself in its inability to invade non-phagocytic cells, to survive in macrophages or to produce death in animal infection models. These effectors could therefore be unequivocally considered factors of virulence. This seems to be the case of

SteA, whose absence significantly attenuated virulence in mouse models of systemic and persistent infection, with failure in spleen colonization (Geddes *et al.*, 2005; Lawley *et al.*, 2006).

Until the year 2005, the gene *steA* (STM1583) was described in the genome of *Salmonella* Typhimurium LT2 as encoding a putative cytoplasmic protein. It was later described that the protein encoded by this gene was secreted by both the SPI-1 and SPI-2 TTSSs, and was named SteA (*Salmonella* translocated effector A) (Geddes *et al.*, 2005). Our bioinformatic analysis shows that the amino acid sequence of SteA is highly conserved among different *S. enterica* subsp. *enterica* serovars, but much less in other *S. enterica* subspecies or in *S. bongori*. This does not only reflect a lower sequence identity, but also the fact that SteA is a longer protein in these *Salmonellae.* In addition, the lack of significant similarity found with proteins from both other *Enterobacteriaceae* and any other bacterial species, indicates that SteA is specific for the genus *Salmonella*. Genes found only in *Salmonella*, may have been acquired after the divergence from other *Enterobacteriaceae* (e.g. *Escherichia coli* or *Klebsiella*), or may have been lost from these genera. Many *S.* Typhimurium genes related with pathogenesis fall in this class, such as SopD and SipA (McClelland *et al.*, 2001)**.** This is also the case of *steA*. In spite of its importance in *S.* Typhimurium virulence, no functional studies specifically focused on SteA had been published at the beginning of this work. Interestingly, SteA of *S.* Typhi, which shows 88% similarity with that of *S.* Typhimurium, was found in a previous work of our group when searching for *Salmonella* effectors that caused growth inhibition when overexpressed in yeast (Alemán *et al.*, 2009). Therefore, we decided to use the *S. cerevisiae* yeast model to try to detect the effects of the expression of *S.* Typhimurium SteA in eukaryotic cells. Although limitations of this model should be taken into consideration, such as the absence of possible posttranscriptional modifications that may occur in the real infection scenario or the fact that the cellular target of the proteins may not be conserved in yeast, it is also noteworthy that in many cases this model has given important clues to understand the function of bacterial effector proteins (Popa *et al.*, 2016).

In our study, we found that the overexpression of *S.* Typhimurium SteA provokes inhibition of yeast cell growth, accompanied by a severe defect in the mitochondrial morphology characterized by the condensation of mitochondria. This study allowed assigning a functional role to the amino-terminal domain of SteA, since we have shown that it was sufficient to induce both growth inhibition and changes in the mitochondrial morphology when overexpressed in yeast cells. One interesting result was that the basic residue K36, required for SteA binding to PI(4)P, was necessary to induce such effects. Thus, these results allowed us to define this N-terminal region, where the K36 residue resides, as a SteA functional region, and suggest that the toxicity of SteA in yeast cells is related to mitochondrial condensation, although we ignore the molecular mechanism. Remarkably, the basic residue (K36) is conserved among *Salmonella enterica* and *bongori*, indicating that this lysine is maintained in even the most divergent species of *Salmonella* and pointing its importance for *Salmonella* SteA function.

Many biochemical and genetic studies in *S. cerevisiae* have shown that mitochondria bind to the actin cytoskeleton, which regulates their morphology, transport and positioning (Anesti & Scorrano, 2006; Boldogh & Pon, 2006). For example, cells treated with the actin-depolymerizing latrunculin-B showed mitochondrial fragmentation and defects in mitochondrial motility (Drubin *et al*., 1993); deletion of the gene encoding the actin-stabilizing protein tropomyosin I affects mitochondrial morphology (Simon *et al*., 1995); and many *act1* actin mutant alleles, whose mitochondrial transport is affected or impaired, also show changes and defects in mitochondrial morphology (Anesti & Scorrano, 2006). However, our results indicate that overexpression of SteA did not seem to specifically disorganize actin in yeast cells, suggesting that the mitochondrial condensation observed is not due to defects in the actin cytoskeleton.

In yeast, mitochondrial morphology and dynamics are controlled by a set of proteins localized in mitochondrial outer (OM), intermediate (IMS) or inner membrane (IM). The mitochore complex (Mmm1, Mdm10 and Mdm12), located at the outer membrane, is one of the classical well-known protein complexes that maintain mitochondrial tubular shape and link mitochondria to the actin cytoskeleton for

movement and inheritance (Boldogh & Pon, 2006). An additional member of the complex, Mdm34, was discovered to tether mitochondria to the ER, and thus, the complex was renamed as ERMES (ER-mitochondria encounter structure) (Kornmann & Walter, 2010; Vevea *et al.*, 2014; AhYoung *et al.*, 2015). A mutation in any of the protein of the mitochore leads to abnormal mitochondria but intact actin cytoskeleton (Boldogh & Pon, 2006), as it happens upon SteA overexpression in yeast. In addition, the inner membrane protein Mdm33 and the dynamin-related GTPase Dnm1, which regulate mitochondrial fission, and the outer membrane protein Gem1 that regulates ERMES complex function, are also responsible for the maintenance of normal mitochondrial morphology (Bleazard *et al.*, 1999; Grimm, 2012; Michel & Kornmann, 2012; AhYoung *et al.*, 2015). As a consequence, their loss causes the network of tubular mitochondrial membranes to collapse, resembling the effect on mitochondria induced by SteA. Those findings together with our results lead us to hypothesize that SteA might be affecting the function of one of the components of the ERMES complex or any cooperating protein, causing the condensation of mitochondria. Interestingly, our data showed that although the overexpression of SteA or SteA$^{(1-97)}$ did not alter ER morphology, SteA$^{(1-97)}$-GFP localized to the ER in yeast.

Mitochondria form a dynamic network within the cell, playing a crucial role in energy metabolism and signaling pathways such as innate immunity or apoptosis, which makes them a target for pathogen interference with host cellular function. Good examples are *Listeria monocytogenes* or *Helicobacter pylori* that induce mitochondrial defects in mammalian cells leading to a loss in the mitochondrial membrane potential (Lebreton *et al*., 2015). As in mammalian cells, loss of selective permeability of the plasma membrane (MMP), loss of mitochondrial membrane potential or production of reactive oxygen species (ROS) are markers related to apoptosis in yeast. Our group has shown, for example, that the growth defect observed when the *Coxiella burnetii* effector CaeA is overexpressed in yeast cells is due to cell death concomitant to loss of MMP and the production of ROS (Rodríguez-Escudero *et al*., 2016). However, our results showed that SteA does not lead to any of the apoptotic-related effects mentioned above, suggesting that it does not seem to elicit pro-apoptotic traits. Therefore, further investigations are needed in order to understand the mechanism behind SteA-induced growth inhibition and mitochondrial morphology changes in yeast cells.

II. The ability of SteA to bind PI(4)P is a determinant of its subcellular localization within yeast cells

In this study, we found that SteA i) localizes to both the PM and vacuole when expressed in *S. cerevisiae*, ii) the PM localization is dependent on the ability of SteA to bind to the phosphoinositide PI(4)P pools generated by the PI 4-kinase Stt4, and iii) the vacuolar localization of SteA also requires the integrity of the Lys36 residue, essential for PI(4)P binding but is independent of typical endosomal phosphoinositides like PI(3)P and PI(3,5)P_2. In parallel to our work, our collaborator Dr. Luis Jaime Mota from ITQB in Lisboa, confirmed by *in vitro* binding assays that SteA is a PI(4)P-binding protein. He also found that interaction with this lipid was necessary for localization of SteA at the PM when ectopically expressed in Hela cells, as well as for its presence at the SCV membrane and Sifs within host cells upon *S.* Typhimurium infection (Domingues *et al*., 2016).

During infection by *Salmonella,* more than 40 effector proteins are secreted and delivered into the host cells by SPI-1 and SPI-2 T3SSs (Niemann *et al.*, 2011; LaRock *et al.*, 2015). Each effector protein secreted during infection exerts its activity depending on its timely delivery and specific localization within host cells (Galan, 2009; Hicks & Galan, 2013). As shown for SteA, other *Salmonella* effectors (SifA, SifB, SopD2, PipB, PipB2, SseF, SseG, SseJ, SseL and SteC) localize both at the Sifs and SCV membrane in mammalian cells (Schroeder *et al.*, 2011), whereas other effector proteins, such as SopB (Patel *et al.*, 2009), SipA (Brawn *et al.*, 2007), SopE and SopE2 (Vonaesch *et al.*, 2014), have only been found at the SCV membrane. Among these effectors, SCV membrane targeting mechanisms had been previously described for SopB (Patel *et al.*, 2009), SopE and SopE2 (Vonaesch *et al.*, 2014), and SifA (Reinicke *et al.*, 2005), but they did not involve binding to phosphoinositides. In addition, the SseI and SspH2 effectors are targeted to the eukaryotic plasma membrane by lipidation mediated by a specific subset of host-cell palmitoyltransferases (Hicks *et al.*, 2011). Therefore, binding to phosphoinositides had not been previously recognized as a mechanism of subcellular targeting of *Salmonella* effectors. However, effectors of other bacterial pathogens are known to exploit host cell phosphoinositides for subcellular targeting, such as *Pseudomonas aeruginosa* effector ExoU that is targeted to the PM by

binding to PI(4,5)P_2 (Gendrin *et al.*, 2012), and several *Legionella* effectors (LpnE, RidL, SetA, LidA, SidC and SidM), which bind to PI(3)P and/or PI(4)P at the LCV (*Legionella*-containing vacuole) membrane (Weber *et al.*, 2006; Ragaz *et al.*, 2008; Brombacher *et al.*, 2009; Weber *et al.*, 2009a; Jank *et al.*, 2012; Finsel *et al.*, 2013). These findings together with our own results lead us to hypothesize that, as shown in the case of SteA, *Salmonella* may target translocated effectors to the PM or the SCV through binding to phosphoinositides.

Phosphoinositides are key regulators of cytoskeleton organization, membrane trafficking and signal transduction. For this reason, they are exploited by pathogens not only to promote specific subcellular localization of bacterial effectors, but to interfere with these essential host cell processes (Weber *et al.*, 2009b). For example, *Legionellae* take advantage of the host PI(3)P and PI(4)P metabolism to generate a permissive environment for their replication. The *Legionella* VipD effector catalyzes the removal of PI(3)P from endosomal membranes due to its phospholipase A_1 activity, blocking fusion with *Legionella*-containing vacuoles (Gaspar & Machner, 2014). Moreover, *Legionella* utilizes initially the host kinase PI4KIIIa to generate PI(4)P on the bacterial PM-derived vacuole to mediate the localization of PI(4)P-binding effectors, as well as the host phosphatase Sac1 for metabolizing the PI(4)P to promote their dissociation later on (Hubber *et al.*, 2014).

The phosphoinositide PI(4)P was initially described to modulate membrane trafficking at the Golgi complex, but it is currently known that PI(4)P has general roles in membrane trafficking and in other cellular processes (De Matteis *et al.*, 2013). In fact, it has been reported that PI(4)P is present not only at the Golgi but also at the PM, secretory vesicles and endocytic compartments in both yeast and mammalian cells (D'Angelo *et al.*, 2008; Szentpetery *et al.*, 2010; De Matteis *et al.*, 2013; Dickson *et al.*, 2014). Moreover, PI4P has been reported to be on a subset of lysosomal membranes under normal conditions, which is required for lysosomal homeostasis (Sridhar *et al.*, 2013), and some evidences support that PI(4)P is also present in the yeast vacuolar membrane (Mayer *et al.*, 2000), predominantly at the vertex ring domain of docked vacuoles (Fratti *et al.*, 2004). Localization of PI(4)P in yeast cells is, therefore, in agreement with that of the three PI 4-kinases, the predominant essential plasma

membrane-localized Stt4 (Cutler *et al.*, 1997; Audhya & Emr, 2002), the Golgi-localized Pik1 (Flanagan *et al.*, 1993), and the non-essential vacuolar/plasma membrane Lsb6 (Han *et al.*, 2002), although this later kinase can be only observed at the vacuolar membrane upon overexpression (Han *et al.*, 2002). Moreover, It has been recently reported that Osh1, one of the members of the oxysterol-binding homology (Osh) protein family that associates with the PI(4)P at the vacuolar membrane, is involved in the counter-transport of this phosphoinositide and ergosterol between the vacuolar and perinuclear ER membranes at the nucleus-vacuole junction (NVJ) (Manik *et al.*, 2017). Therefore, the vacuolar localization of SteA, particularly at the vacuole-to-vacuole contact areas for homotypic fusion, might be dependent on the vacuolar membrane PI(4)P pool.

When we used the PI(4)P-binding domain (P4C) of the *Legionella* effector SidC to check PI(4)P subcellular localization in yeast, we found that the P4C probe localized preferentially at the Golgi and was also observed at the PM, in agreement with the literature, but not at the vacuole. The same result was obtained by our collaborator Dr. Mota by using this probe in HeLa cells (Domingues *et al.*, 2016). Interestingly, the P4C probe co-localized with SteA at the PM, which confirmed the binding activity of this *Salmonella* effector to the PI(4)P pool present at this structure, but neither at the Golgi (only marked by P4C) nor at the vacuole (only marked by SteA) in yeast cells. The individual localization experiments also showed that the GFP-SteA fluorescence signal at the PM was more intense than that of P4C-mCherry, suggesting a higher affinity of SteA for PI(4)P at this subcellular location. Therefore, these two bacterial derived probes could be used in yeast cells to separate the two pools of PI(4)P dependent on Stt4 and Pik1 at the plasma membrane and Golgi, respectively. A similar result was obtained in HeLa cells, in which SteA localizes at some PI(4)P-enriched environments marked with P4C (PM, SCV membrane and Sifs) but not at others (Golgi complex) (Domingues *et al.*, 2016). These results indicate that the SidC P4C domain and SteA display distinct preferences for binding PI(4)P at different locations and suggest that binding of SteA to Golgi PI(4)P may need additional, yet unidentified, factors.

One surprising finding was that cells showed more PM-localized P4C probe when it is co-expressed with SteA than when expressed alone. The regulation of the

lipid-signaling molecule PI(4)P at the PM is controlled by the oxysterol-binding homology (Osh) and the integral ER VAP proteins through activation of the ER-resident Sac1 PI(4)P phosphatase. Loss of Sac1 in mammalian cells led to a very robust increase in GFP-P4CSidC signal both at the plasma membrane and intracellular membranes (Dong *et al.*, 2016), and in yeast caused an accumulation of PI(4)P at the PM, ER and vacuolar membranes (Roy & Levine, 2004; Tahirovic *et al.*, 2005). According to this, elimination of either Osh or VAP proteins results in a 3- to 7-fold increase in PI(4)P levels in yeast cells (English & Voeltz, 2013). Taken these findings into account, our results suggest that SteA might be increasing PI(4)P levels at the PM in yeast cells through inhibition of Sac1 activity either directly or indirectly by inhibiting the function of Osh proteins. The Osh family in yeast is composed of seven proteins that bind PI(4)P in different locations and are involved in lipid transport between membranes (Tong *et al.*, 2016). Among them, Osh2p, Osh3p, Osh6p and Osh7p are found in the cell periphery, at putative ER-PM contact sites. Therefore, it is plausible to hypothesize that SteA may compete with Osh proteins for binding to this phosphoinositide at these sites, hindering Sac1 activation. This could increase binding of the P4C probe to the PM as well as accumulation of SteA itself both at the PM and the vacuole.

The amino acid sequence of SteA does not display significant similarity either to known eukaryotic PI(4)P binding domains (Kutateladze, 2010) or the PI(4)P binding domains of *Legionella* effectors SidC and SidM (Ragaz *et al.*, 2008; Brombacher *et al.*, 2009). However, in spite of the general low sequence identity among phosphoinositide-binding domains, they commonly show strong positive electrostatic potential located on the phosphoinositide-binding surface (Cho & Stahelin, 2005). For example, the SidC P4C domain includes a positively charged pocket with two essential arginines at the binding site for PI(4)P (Luo *et al.*, 2015). Other proteins, such as eukaryotic profilin or Wiskott-Aldrich syndrome protein (WASP), recognize and bind to the phosphoinositide PI(4,5)P_2 through clusters of basic residues (Kutateladze, 2010). In agreement to this, although we have not defined the precise PI(4)P-binding domain within SteA, we have demonstrated that the association of SteA to this phosphoinositide at the yeast PM and vacuole was dependent on a lysine-rich region, and particularly on the K36 residue, within a predicted α-helix located at its N-terminal domain. Interestingly, the PI(4)P-

and oxysterol-binding protein Osh4 possesses a surface basic region that binds anionic lipids (Von Filseck *et al*., 2015). Similarly to the mutation of K36 of SteA, a mutation in the lysine 336 (K336A) within this region of Osh4 abolished both its binding to PI(4)P and its sterol/PI(4)P exchange activity. This data support our idea that SteA might be competing with Osh proteins for binding to PI(4)P.

The SteA K36 residue has been shown by our collaborator Dr. Mota to be required for *in vitro* binding of SteA to PI(4)P, as well as for localization of SteA both at the PM when ectopically expressed in HeLa cells and at the SCV membrane and Sifs in infected HeLa cells (Domingues *et al.*, 2016). Further detailed studies of structure and function of the PI(4)P binding activity of SteA are required to understand structural details of how phosphoinositide recognition occurs in this case. As discussed above, in addition to these findings, we found that the K36 residue was also necessary for SteA-induced yeast growth inhibition and mitochondrial condensation, confirming that this lysine key to the function of SteA and that the toxic effect of this bacterial effector in yeast could be related to its interaction with negatively charged lipids in eukaryotic cellular membranes. However, in spite of its mis-localization in HeLa cells, the $SteA^{K36A}$ mutant protein still complemented the *ΔsteA Salmonella* mutant phenotypes related to the maintenance of SCV during infection (Domingues *et al.*, 2016). This suggests that PI(4)P binding would not be necessary for the previously described contribution of SteA to the control of SCV dynamics (Domingues *et al.*, 2014), but likely required for other cellular functions of SteA during host cell infection.

Recent work on the communication among cellular organelles has revealed the existence of a membrane contact site between vacuoles and mitochondria, named vCLAMP (vacuole and mitochondria patch) (Elbaz-Alon *et al.*, 2014). This site is marked by Vps39, a component of the homotypic fusion and vacuole protein sorting (HOPS) tethering complex (Stroupe *et al.*, 2006). These authors showed that the vCLAMP and the above mentioned ER-mitochondria tethering complex ERMES are co-regulated and work in parallel in lipid transport between the endomembrane system and mitochondria. The absence of one of these contact sites causes expansion of the other and elimination of both is lethal, indicating that mitochondria are depending on having at least one of them. Therefore, it is tempting to speculate that the possible

effects of SteA on vacuolar membrane lipids could affect vCLAMP, altering its equilibrium with ERMES and eventually leading to the observed mitochondrial condensation.

In sum, we have found that SteA localizes to the yeast PM and vacuole and that binding of SteA to PI(4)P is a determinant of its localization within yeast cell. Further studies are needed to clarify if subcellular targeting by phosphoinositide-binding is common to several *Salmonella* effectors and to better understand the effect of SteA binding to PI(4)P in yeast. Indeed, the mechanisms determining the membrane specificity of the PI(4)P binding pocket of SteA is an interesting problem remaining to be solved. Nevertheless, our results are sufficient to indicate that SteA is the first known *Salmonella* PI(4)P-binding effector.

III. SteA requires vacuolar protein sorting proteins in order to localize to the yeast vacuole

In *S. cerevisiae*, proteins translocated to the ER are directed to the Golgi apparatus where they are sorted between those targeted to the PM or to the external medium via the secretory pathway and those targeted to the vacuole either directly through the ALP pathway or through endosomes via the VPS/CPY pathway. PM proteins can be internalized by endocytosis and transported to endosomes where they are sorted either to the vacuole or redirected to the Golgi via the RCY pathway where they enter the secretory pathway to be redirected to the PM (Feyder *et al*., 2015). To date, more than 60 *VPS* genes have been described, based on the vacuolar morphology of the corresponding mutants (Bowers & Stevens, 2005). Mapping SteA in different *vps* mutants showed us that SteA is one of those proteins that need the presence of particular Vps proteins in order to localize to the yeast vacuole.

Vps3 and Vps8 are specific subunits of the CORVET complex that interact with the Rab5 homolog Vps21 and assure the fusion of TGN derived vesicles to endosomal membranes (Peplowska *et al.*, 2007). The corresponding *vps3* and *vps8* mutants, as well as *vps45*, belong to the class D of *vps* mutants, characterized for having a single enlarged vacuole (Raymond *et al*., 1992). Most of class D mutants also showed an accumulation of small vesicles throughout the cytoplasm that are unable to fuse with

LE/MVB (Markgraf *et al.*, 2009). The MVB machinery ensures the delivery of proteins to the vacuolar lumen by fusion of the MVB and the vacuole and it is composed of four complexes named ESCRT (0, I, II and III), whose components belong to class E Vps proteins. Mutants in these proteins show a large aberrant late endosome/MVB adjacent to the vacuole, named class E compartment, where vacuolar proteins accumulate due to a blockage in their transport to the vacuolar lumen (Bowers & Stevens, 2005; Henne *et al.*, 2011). Our data showed that SteA fails to localize to the vacuolar lumen in both class D and E *vps* mutants, only faintly appearing at the vacuolar membrane where it accumulates in large puncta. However, while in class E mutants SteA localizes in typical FM4-64-marked class E compartments, the SteA-containing dots observed in class D mutants are not stained with the endosomal marker FM4-64. All these results indicate that SteA is delivered to the vacuole via MVB sorting. However, the nature of the puncta in which SteA accumulates in class D mutants is intriguing. It has been reported that some components of the HOPS (homotypic fusion and vacuole protein sorting) complex, like Vps39, localize to specific patches at the vacuolar membrane, which correspond to the vCLAMP. Since Vps39 also accumulate at the vertex ring during vacuolar docking (Wang *et al.*, 2002), where SteA is recruited as well, one plausible hypothesis is that the contact site between vacuole and mitochondria could be also enriched for SteA. Further experiments should be required to validate this hypothesis. A second challenge that these results pose is that, as SteA vacuolar localization requires the CORVET/HOPS pathway, our hypothesis that PI(4)P could also be a determinant for SteA tethering to vacuolar membranes would imply that the appearance of pools of this lipid in such compartment should in turn depend on an intact Vps pathway. This particular point has not been investigated in depth, to our knowledge.

IV. Overexpression of SteA shows genetic interactions with mutations in genes related to vacuole homeostasis

Fab1 phosphoinositide kinase is one of the PI(3)P effectors, using this phosphoinositide species as a substrate to generate discrete pools of $PI(3,5)P_2$, involved in the fusion of the MVB with the vacuole (Mayinger, 2012). Vac14 is the scaffold protein that nucleates the assembly of the Fab1 complex at the vacuole (Jin *et al.*, 2008;

Dove *et al.*, 2009). Our results show that SteA overexpression displayed a negative genetic interaction with both *fab1*Δ and *vac14*Δ mutants. However, subcellular localization of SteA is not affected by loss of any of the Fab1 complex proteins, and no obvious direct physical interaction was detected by the co-IP strategy we used. These results led us to hypothesized that, although the presence of $PI(3,5)P_2$ is not needed to target SteA toward the vacuole, SteA might be functionally related with the Fab1 complex. In agreement, we also found a phenotypic enhancement of SteA toxicity in 4 out of the 11 mutants (*vps3*, *vps1*, *cla4* or *vps35*) known as negative genetic interactors of *vac14* and *fab1* mutants. Of remark, one of these mutants lacks the class D Vps3 protein, also shown in our work to be required for proper localization of SteA at the vacuole, and other was depleted of Cla4, a p21-activated protein kinase (PAK) involved in cell polarity regulation that binds PI(4)P in yeast (Santiago-Tirado & Bretscher, 2011). In the event that SteA is interfering with putative PI(4)P pools or their effectors at vacuolar membranes, it is likely that the presence of PI(3,5)P2 is necessary to somehow compensate for SteA-induced damage and maintain viability.

Among mutants reported to show negative genetic interaction, like SteA overproduction does, with *vps3*, *vps1*, *cla4* or *vps35* mutants, we found that deletions in *VMA1* (coding for a component of the vacuolar v-ATPase), *VMA21* (coding for a protein involved in v-ATPase assembly and function), or *DYN3* (subunit of the dynein complex related to nuclear migration) also enhanced the toxicity caused by SteA overexpression. Interestingly, we found that a v-ATPase-related mutant, *voa1*Δ, was the only mutant showing phenotypic suppression for SteA-induced toxicity. Voa1 is the assembly factor of the V0 sector to the vacuolar ATPase, and it is localized at the ER of *S. cerevisiae* (Ryan *et al.*, 2008). Independent of its role as a cellular proton pump, the V0 sector has been suggested to function in membrane fusion and vesicle formation (Wada *et al.*, 2008). Further investigations are needed in order to understand why SteA toxicity in yeast depends on the presence of Voa1, but it seems that it would be more likely related to the role of the v-ATPase as putative regulator of vacuolar morphology and membrane trafficking than to its classical pump activity.

CONCLUSIONS

1. SteA is highly conserved among different *S. enterica* serovars, and it is specific for the *Salmonella* genus.

2. Overexpression of SteA in yeast provokes a significant growth inhibition dependent on its amino-terminal region.

3. SteA overexpression leads to defects in the mitochondrial morphology, characterized by the appearance of condensed mitochondria. The amino-terminal region of SteA is necessary and sufficient to cause this mitochondrial phenotype.

4. SteA localizes at the plasma membrane and vacuole when expressed in *S. cerevisiae*, whereas the amino-terminal fragment corresponding to the first 97 amino acids of SteA localizes to the ER.

5. The plasma membrane localization of SteA requires the presence of PI(4)P pools generated by the PM-associated PI 4-kinase Stt4.

6. Tethering SteA to the plasma membrane and vacuole in *S. cerevisiae* requires basic residues near its N-terminus, specifically Lys36. This basic residue is also necessary to induce both growth inhibition and mitochondrial condensation in yeast cells.

7. Class D and E Vps proteins are necessary for vacuolar localization of SteA, pointing to the importance of sorting at late endosome/MVB in SteA targeting to the vacuole.

8. Mutants lacking PI(3)P 5-kinase complex subunits Vac14 and Fab1, show a negative genetic interaction with SteA overexpression.

9. Mutations in *CLA4*, *VPS1*, *VPS3* and *VPS35* cause phenotypic enhancement of SteA-induced growth inhibition.

10. The absence of Voa1, an ER protein that functions in assembly of the V0 sector of V-ATPase, but not that of other V-ATPase subunits, suppresses SteA-induced growth inhibition in *S. cerevisiae*.

BIBLIOGRAPHY

Abrahams, G.L., Muller, P., and Hensel, M. (2006) Functional dissection of SseF, a type III effector protein involved in positioning the *Salmonella*-containing vacuole. *Traffic* **7**: 950-965.

AhYoung, A.P., Jiang, J., Zhang, J., Khoi Dang, X., Loo, J.A., Zhou, Z.H., and Egea, P.F. (2015) Conserved SMP domains of the ERMES complex bind phospholipids and mediate tether assembly. *Proc Natl Acad Sci U S A* **112**: E3179-3188.

Alemán, A., Rodríguez-Escudero, I., Mallo, G.V., Cid, V.J., Molina, M., and Rotger, R. (2005) The amino-terminal non-catalytic region of Salmonella typhimurium SigD affects actin organization in yeast and mammalian cells. *Cell Microbiol* **7**: 1432-1446.

Aleman, A., Fernandez-Pinar, P., Perez-Nunez, D., Rotger, R., Martin, H., and Molina, M. (2009) A yeast-based genetic screen for identification of pathogenic *Salmonella* proteins. *FEMS Microbiol Lett* **296**: 167-177.

Alemán, A., Rodríguez-Escudero, I., Mallo, G.V., Cid, V.J., Molina, M., and Rotger, R. (2005) The amino-terminal non-catalytic region of *Salmonella* typhimurium SigD affects actin organization in yeast and mammalian cells. *Cell Microbiol* **7**: 1432-1446.

Alexander F. A. Keszei , X.T., Craig McCormick, Elton Zeqiraj, John R. Rohde, Mike Tyers, Frank Sicheri (2014) Structure of an SspH1-PKN1 Complex Reveals the Basis for Host Substrate Recognition and Mechanism of Activation for a Bacterial E3 Ubiquitin Ligase *Mol Cell Biol* **34**: 362-373.

Andres-Pons, A., Rodríguez-Escudero, I., Gil, A., Blanco, A., Vega, A., Molina, M., Pulido, R., and Cid, V.J. (2007) In vivo functional analysis of the counterbalance of hyperactive phosphatidylinositol 3-kinase p110 catalytic oncoproteins by the tumor suppressor PTEN. *Cancer Res* **67**: 9731-9739.

Anesti, V., and Scorrano, L. (2006) The relationship between mitochondrial shape and function and the cytoskeleton. *Biochim Biophys Acta* **1757**: 692-699.

Audhya, A., and Emr, S.D. (2002) Stt4 PI 4-Kinase Localizes to the Plasma Membrane and Functions in the Pkc1-Mediated MAP Kinase Cascade. *Dev Cell* **2**: 593–605.

Audhya, A., Foti, M., and and Emr, S.D. (2000) Distinct Roles for the Yeast Phosphatidylinositol 4-Kinases, Stt4p and Pik1p, in Secretion, Cell Growth, and Organelle Membrane Dynamics. *Mol Biol Cell* **11**: 2673–2689.

Auluck, A.K., Caraveo, G., and Lindquist, S. (2010) α-Synuclein: Membrane Interactions and Toxicity in Parkinson's Disease *Annu Rev Cell Dev Biol* **26**:211-233.

Baison-Olmo, F., Cardenal-Munoz, E., and Ramos-Morales, F. (2012) PipB2 is a substrate of the *Salmonella* pathogenicity island 1-encoded type III secretion system. *Biochem Biophys Res Commun* **423**: 240-246.

Bakowski, M.A., Braun, V., and Brumell, J.H. (2008) *Salmonella*-containing vacuoles:

directing traffic and nesting to grow. *Traffic* **9**: 2022-2031.

Bakowski, M.A., Braun, V., Lam, G.Y., Yeung, T., Heo, W.D., Meyer, T., Finlay, B.B., Grinstein, S., and Brumell, J.H. (2010) The phosphoinositide phosphatase SopB manipulates membrane surface charge and trafficking of the *Salmonella*-containing vacuole. *Cell Host Microbe* **7**: 453-462.

Bakowski, M.A., Cirulis, J.T., Brown, N.F., Finlay, B.B., and Brumell, J.H. (2007) SopD acts cooperatively with SopB during *Salmonella* enterica serovar Typhimurium invasion. *Cell Microbiol* **9**: 2839-2855.

Bakshi, C.S., Singh, V.P., Wood, M.W., Jones, P.W., Wallis, T.S., and Galyov, E.E. (2000) Identification of SopE2, a *Salmonella* Secreted Protein Which Is Highly Homologous to SopE and Involved in Bacterial Invasion of Epithelial Cells. *J Bacteriol* **182**: 2341-2344.

Bardwell, L. Cook, J.G., Zhu-Shimoni, J.X., Voora, D., and Thorner, J. (1998) Differential regulation of transcription: repression by unactivated mitogen-activated protein kinase Kss1 requires the Dig1 and Dig2 proteins. *Proc Natl Acad Sci* U S A **95**(**26**):15400-5.

Barlow, C.A., Laishram, R.S., and Anderson, R.A. (2010) Nuclear phosphoinositides: a signaling enigma wrapped in a compartmental conundrum. *Trends Cell Biol* **20**: 25-35.

Bayer-Santos, E., Durkin, C.H., Rigano, L.A., Kupz, A., Alix, E., Cerny, O., Jennings, E., Liu, M., Ryan, A.S., Lapaque, N., Kaufmann, S.H., and Holden, D.W. (2016) The Salmonella Effector SteD Mediates MARCH8-Dependent Ubiquitination of MHC II Molecules and Inhibits T Cell Activation. *Cell Host Microbe* **20**: 584-595.

Beh, C.T., McMaster, C.R., Kozminski, K.G., and Menon, A.K. (2012) A detour for yeast oxysterol binding proteins. *J Biol Chem* **287**: 11481-11488.

Bernal-Bayard, J., Cardenal-Munoz, E., and Ramos-Morales, F. (2010) The *Salmonella* type III secretion effector, *Salmonella* leucine-rich repeat protein (SlrP), targets the human chaperone ERdj3. *J Biol Chem* **285**: 16360-16368.

Bernal-Bayard, J., and Ramos-Morales, F. (2009) *Salmonella* type III secretion effector SlrP is an E3 ubiquitin ligase for mammalian thioredoxin. *J Biol Chem* **284**: 27587-27595.

Bhavsar, A.P., Brown, N.F., Stoepel, J., Wiermer, M., Martin, D.D., Hsu, K.J., Imami, K., Ross, C.J., Hayden, M.R., Foster, L.J., Li, X., Hieter, P., and Finlay, B.B. (2013) The *Salmonella* type III effector SspH2 specifically exploits the NLR co-chaperone activity of SGT1 to subvert immunity. *PLoS Pathog* **9**: e1003518.

Birkeland, H.C., and Stenmark, H. (2004) Protein targeting to endosomes and phagosomes via FYVE and PX domains. *Curr Top Microbiol Immunol* **282**: 89-115.

Bleazard, W., McCaffery, J.M., King, E.J., Bale, S., Mozdy, A., Tieu, Q., Nunnari, J., and Shaw, J.M. (1999) The dynamin-related GTPase Dnm1 regulates mitochondrial fission in yeast. *Nat Cell Biol* **1**: 298-304.

Boldogh, I.R., and Pon, L.A. (2006) Interactions of mitochondria with the actin cytoskeleton. *Biochim Biophys Acta* **1763**: 450-462.

Boman, A.L., Zhang, C.-J., Zhu, X., and and Kahn, R.A. (2000) A Family of ADP-Ribosylation Factor Effectors That Can Alter Membrane Transport through the trans-Golgi. *Mol Biol Cell* **11**: 1241–1255.

Boone, C., Bussey, H., and Andrews, B.J. (2007) Exploring genetic interactions and networks with yeast. *Nat Rev Genet* **8**: 437-449.

Botelho, R.J., Efe, J.M., Teis, D., and and Emr, S.D. (2008) Assembly of a Fab1 Phosphoinositide Kinase Signaling Complex Requires the Fig4 Phosphoinositide Phosphatase. *Mol Biol Cell* **19**: 4273–4286.

Boucrot, E., Beuzon, C.R., Holden, D.W., Gorvel, J.P., and Meresse, S. (2003) *Salmonella* typhimurium SifA effector protein requires its membrane-anchoring C-terminal hexapeptide for its biological function. *J Biol Chem* **278**: 14196-14202.

Boucrot, E., Henry, T., Borg, J.P., Gorvel, J.P., and Méresse, S. (2005) The intracellular fate of *Salmonella* depends on the recruitment of kinesin. *Science* **308**: 1174-1178.

Bowers, K., and Stevens, T.H. (2005) Protein transport from the late Golgi to the vacuole in the yeast Saccharomyces cerevisiae. *Biochim Biophys Acta* **1744**: 438-454.

Boyle, E.C., Bishop, J.L., Grassl, G.A., and Finlay, B.B. (2007) *Salmonella*: from pathogenesis to therapeutics. *J Bacteriol* **189**: 1489-1495.

Boyle, E.C., Brown, N.F., and Finlay, B.B. (2006) *Salmonella* enterica serovar Typhimurium effectors SopB, SopE, SopE2 and SipA disrupt tight junction structure and function. *Cell Microbiol* **8**: 1946-1957.

Brawn, L.C., Hayward, R.D., and Koronakis, V. (2007) Salmonella SPI1 effector SipA persists after entry and cooperates with a SPI2 effector to regulate phagosome maturation and intracellular replication. *Cell Host Microbe* **1**: 63-75.

Brombacher, E., Urwyler, S., Ragaz, C., Weber, S.S., Kami, K., Overduin, M., and Hilbi, H. (2009) Rab1 guanine nucleotide exchange factor SidM is a major phosphatidylinositol 4-phosphate-binding effector protein of Legionella pneumophila. *J Biol Chem* **284**: 4846-4856.

Brumell , J.H., Goosney, D.L., and Finlay, B.B. (2002) SifA, a Type III Secreted Effector of *Salmonella* typhimurium,Directs*Salmonella*-Induced Filament (Sif) Formation Along Microtubules. *Traffic* **3**: 407-415.

Brumell, J.H., Kujat-Choy, S., Brown, N.F., Vallance, B.A., Knodler, L.A., and Finlay, B.B. (2003) SopD2 is a Novel Type III Secreted Effector of *Salmonella* typhimurium That Targets Late Endocytic Compartments Upon Delivery Into Host Cells. *Traffic* **4**: 36-48.

Brumell, J.H., Rosenberger, C.M., Gotto, G.T., Marcus, S.L., and Finlay, B.B. (2001) SifA permits survival and replication of *Salmonella* typhimurium in murine macrophages. *Cellular Microbiology* **3(2)**: 75-84.

Bryant, N.J., Piper, R.C., Weisman, L.S., and Stevens, T.H. (1998) Retrograde Traffic Out of the Yeast Vacuole to the TGN Occurs via the Prevacuolar/Endosomal Compartment. *The Journal of Cell Biology* **142**: 651–663.

Burstein, D., Satanower, S., Simovitch, M., Belnik, Y., Zehavi, M., Yerushalmi, G., Ben-Aroya, S., Pupko, T., and Banin, E. (2015) Novel type III effectors in Pseudomonas aeruginosa. *MBio* **6**: e00161.

Campodonico, E.M., Chesnel, L., and Roy, C.R. (2005) A yeast genetic system for the identification and characterization of substrate proteins transferred into host cells by the Legionella pneumophila Dot/Icm system. *Mol Microbiol* **56**: 918-933.

Cardenal-Munoz, E., Gutierrez, G., and Ramos-Morales, F. (2014) Global impact of *Salmonella* type III secretion effector SteA on host cells. *Biochem Biophys Res Commun* **449**: 419-424.

Cardenal-Munoz, E., and Ramos-Morales, F. (2011) Analysis of the expression, secretion and translocation of the *Salmonella* enterica type III secretion system effector SteA. *PLoS One* **6**: e26930.

Cho, W., and Stahelin, R.V. (2005) Membrane-protein interactions in cell signaling and membrane trafficking. *Annu Rev Biophys Biomol Struct* **34**: 119-151.

Cid, V.J., Rodríguez-Escudero, I., Andres-Pons, A., Roma-Mateo, C., Gil, A., den Hertog, J., Molina, M., and Pulido, R. (2008) Assessment of PTEN tumor suppressor activity in nonmammalian models: the year of the yeast. *Oncogene* **27**: 5431-5442.

Clague, M.J., and Urbe, S. (2008) Multivesicular bodies. *Curr Biol* **18**: R402-404.

Conibear, E. (2010) Converging views of endocytosis in yeast and mammals. *Curr Opin Cell Biol* **22**: 513-518.

Coombes, B.K., Lowden, M.J., Bishop, J.L., Wickham, M.E., Brown, N.F., Duong, N., Osborne, S., Gal-Mor, O., and Finlay, B.B. (2007) SseL is a *Salmonella*-specific translocated effector integrated into the SsrB-controlled *Salmonella* pathogenicity island 2 type III secretion system. *Infect Immun* **75**: 574-580.

Coombes, B.K., Wickham, M.E., Brown, N.F., Lemire, S., Bossi, L., Hsiao, W.W., Brinkman, F.S., and Finlay, B.B. (2005) Genetic and molecular analysis of GogB, a phage-encoded type III-secreted substrate in *Salmonella* enterica serovar typhimurium with autonomous expression from its associated phage. *J Mol Biol*

348: 817-830.

Cowles, C.R., Snyder, W.B., Burd, S.G., and and Emr, S.D. (1997) Novel Golgi to vacuole delivery pathway in yeast: identification of a sorting determinant and required transport component. *EMBO J* **16**: 2769–2782.

Cullen, P.J., Cozier, G.E., Banting, G., and Mellor, M. (2001) Modular phosphoinositide-binding domains – their role in signalling and membrane trafficking. *Current Biology* **11**: R882–R893.

Curak, J., Rohde, J., and Stagljar, I. (2009) Yeast as a tool to study bacterial effectors. *Curr Opin Microbiol* **12**: 18-23.

Cutler, N.S., Heitman, J., and Cardenas, M.E. (1997) STT4 Is an Essential Phosphatidylinositol 4-Kinase That Is a Target of Wortmannin in Saccharomyces cerevisiae. *J Biol Chem*: 44.

D'Angelo, G., Vicinanza, M., Di Campli, A., and De Matteis, M.A. (2008) The multiple roles of PtdIns(4)P- not just the precursor of PtdIns(4,5) 2. *J Cell Sci* **121**.

D'Costa, V.M., Braun, V., Landekic, M., Shi, R., Proteau, A., McDonald, L., Cygler, M., Grinstein, S., and Brumell, J.H. (2015) *Salmonella* Disrupts Host Endocytic Trafficking by SopD2-Mediated Inhibition of Rab7. *Cell Rep* **12**: 1508-1518.

De Matteis, M.A., Wilson, C., and D'Angelo, G. (2013) Phosphatidylinositol-4-phosphate: the Golgi and beyond. *Bioessays* **35**: 612-622.

Deiwick, J., Salcedo, S.P., Boucrot, E., Gilliland, S.M., Henry, T., Petermann, N., Waterman, S.R., Gorvel, J.P., Holden, D.W., and Meresse, S. (2006) The translocated *Salmonella* effector proteins SseF and SseG interact and are required to establish an intracellular replication niche. *Infect Immun* **74**: 6965-6972.

Deleu, S., Choi, K., Reece, J.M., and Shears, S.B. (2006) Pathogenicity of *Salmonella*: SopE-mediated membrane ruffling is independent of inositol phosphate signals. *FEBS Lett* **580**: 1709-1715.

Derre, I., and Isberg, R.R. (2005) LidA, a translocated substrate of the Legionella pneumophila type IV secretion system, interferes with the early secretory pathway. *Infect Immun* **73**: 4370-4380.

Desrivieres, S., Cooke, F.T., Parker, P.J., and Hall, M.N. (1998) MSS4, a Phosphatidylinositol-4-phosphate 5-Kinase Required for Organization of the Actin Cytoskeleton in Saccharomyces cerevisiae. *The Journal of Biological Chemistry* **273**: 15787–15793.

Di Ventura, B., Funaya, C., Antony, C., Knop, M., and Serrano, L. (2008) Reconstitution of Mdm2-dependent post-translational modifications of p53 in yeast. *PLoS One* **3**: e1507.

Dickson, E.J., Jensen, J.B., and Hille, B. (2014) Golgi and plasma membrane pools of PI(4)P contribute to plasma membrane PI(4,5)P2 and maintenance of KCNQ2/3

ion channel current. *Proc Natl Acad Sci U S A* **111**: E2281-2290.

Diepold, A., and Wagner, S. (2014) Assembly of the bacterial type III secretion machinery. *FEMS Microbiol Rev* **38**: 802-822.

Divecha, N., and Halstead, J.R. (2004) Of yeast and men. The evolution of PtdIns(3,4,5)P(3) synthesis. *EMBO Rep* **5**: 865-866.

Domingues, L., Holden, D.W., and Mota, L.J. (2014) The *Salmonella* effector SteA contributes to the control of membrane dynamics of *Salmonella*-containing vacuoles. *Immu infect* **82**: 2923–2934.

Domingues, L., Ismail, A., Charro, N., Rodríguez-Escudero, I., Holden, D.W., Molina, M., Cid, V.J., and Mota, L.J. (2016) The *Salmonella* effector SteA binds phosphatidylinositol 4-phosphate for subcellular targeting within host cells. *Cell Microbiol* **18**: 949-969.

Dong H, Z.X., Pan Z, Peng D, Liu X. (2008) Identification of genes for biofilm formation in a *Salmonella* enteritidis strain by transposon mutagenesis. *Wei Sheng Wu Xue Bao* **48**: 869-873.

Dong, R., Saheki, Y., Swarup, S., Lucast, L., Harper, J.W., and De Camilli, P. (2016) Endosome-ER Contacts Control Actin Nucleation and Retromer Function through VAP-Dependent Regulation of PI4P. *Cell* **166**: 408-423.

Dove, S.K., Dong, K., Kobayashi, T., Williams, F.K., and Michell, R.H. (2009) Phosphatidylinositol 3,5-bisphosphate and Fab1p/PIKfyve underPPIn endo-lysosome function. *Biochem J* **419**: 1-13.

Dove, S.K., McEwen, R.K., Mayes, A., Hughes, D.C., Beggs, J.D., and and Michell, R.H. (2002) Vac14 Controls PtdIns(3,5)P2 Synthesis and Fab1-Dependent Protein Trafficking to the Multivesicular Body. *Current Biology* **12** 885–893.

Drubin, D.G., Jones, H.D., and and Wertman, K.F. (1993) Actin Structure and Function: Roles in Mitochondrial Organization and Morphogenesis in Budding Yeast and Identification of the Phalloidin-binding Site. *Mol Biol Cell* **4**: 1277-1294.

Du, F., and Galan, J.E. (2009) Selective inhibition of type III secretion activated signaling by the *Salmonella* effector AvrA. *PLoS Pathog* **5**: e1000595.

Duina, A.A., Miller, M.E., and Keeney, J.B. (2014) Budding yeast for budding geneticists: a primer on the Saccharomyces cerevisiae model system. *Genetics* **197**: 33-48.

Duncan, M.C., Costaguta, G., and and Payne, G.S. (2003) Yeast epsin-related proteins required for Golgi–endosome traffic define a γ-adaptin ear-binding motif. *Nat Cell Biol* **5**: 77 - 81.

Edward A. Miao, M.B., Samuel I. Miller, Robert L. Jeng, Andrea Haraga ,Matthew D. Welch (2003) *Salmonella* effectors translocated across the vacuolar membrane interact with the actin cytoskeleton. *Mol Cell Biol* **48**: 401-415.

Eisenberg, T., Carmona-Gutierrez, D., Buttner, S., Tavernarakis, N., and Madeo, F. (2010) Necrosis in yeast. *Apoptosis* **15**: 257-268.

Elbaz-Alon, Y., Rosenfeld-Gur, E., Shinder, V., Futerman, A.H., Geiger, T., and Schuldiner, M. (2014) A dynamic interface between vacuoles and mitochondria in yeast. *Dev Cell* **30**: 95-102.

English, A.R., and Voeltz, G.K. (2013) Endoplasmic reticulum structure and interconnections with other organelles. *Cold Spring Harb Perspect Biol* **5**: a013227.

Espinoza, R.A., Silva-Valuenzuela, C.A., Amaya, F.A.,. Urrutia, Í.M., Contreras, I., and Santiviago, C.A. (2017) Differential roles for pathogenicity islands SPI-13 and SPI-8 in the interaction of *Salmonella* Enteritidis and *Salmonella* Typhi with murine and human macrophages. *Biol Res* **50**: 5.

Fàbrega, A., and Vila, J. (2013) *Salmonella* enterica serovar Typhimurium skills to succeed in the host: virulence and regulation. *Clin Microbiol Rev* **26**: 308-341.

Fairn, G.D., Hermansson, M., Somerharju, P., and Grinstein, S. (2011) Phosphatidylserine is polarized and required for properCdc42 localizationand for development of cellpolarity. *Nat Cell Biol* **13**:1424–1430.

Fernandez-Acero, T., Rodríguez-Escudero, I., Molina, M., and Cid, V.J. (2015) The yeast cell wall integrity pathway signals from recycling endosomes upon elimination of phosphatidylinositol (4,5)-bisphosphate by mammalian phosphatidylinositol 3-kinase. *Cell Signal* **27**: 2272-2284.

Fernandez-Pinar, P., Aleman, A., Sondek, J., Dohlman, H.G., Molina, M., and Martin, H. (2012) The *Salmonella* Typhimurium effector SteC inhibits Cdc42-mediated signaling through binding to the exchange factor Cdc24 in Saccharomyces cerevisiae. *Mol Biol Cell* **23**: 4430-4443.

Feyder, S., De Craene, J.O., Bar, S., Bertazzi, D.L., and Friant, S. (2015) Membrane trafficking in the yeast Saccharomyces cerevisiae model. *Int J Mol Sci* **16**: 1509-1525.

Figueira, R., and Holden, D.W. (2012) Functions of the *Salmonella* pathogenicity island 2 (SPI-2) type III secretion system effectors. *Microbiology* **158**: 1147-1161.

Figueira, R., Watson, K.G., Holden, D.W., and Helaine, S. (2013) Identification of *Salmonella* pathogenicity island-2 type III secretion system effectors involved in intramacrophage replication of S. enterica serovar typhimurium: implications for rational vaccine design. *MBio* **4**: e00065.

Finsel, I., Ragaz, C., Hoffmann, C., Harrison, C.F., Weber, S., van Rahden, V.A., Johannes, L., and Hilbi, H. (2013) The Legionella effector RidL inhibits retrograde trafficking to promote intracellular replication. *Cell Host Microbe* **14**: 38-50.

Flanagan, C.A., Schnieders, E.A., Emerick, A.W., Kunisawa, R., Admon, A., and Thorner, J. (1993) Phosphatidylinositol 4-Kinase: Gene Structure and Requirement for Yeast Cell Viability. *Science* **262**: 1444-1448.

Foti, M., Audhya, A., and and Emr, S.D. (2001) Sac1 Lipid Phosphatase and Stt4 Phosphatidylinositol 4-Kinase Regulate a Pool of Phosphatidylinositol 4-Phosphate That Functions in the Control of the Actin Cytoskeleton and Vacuole Morphology. *Mol Biol Cell* **12**: 2396–2411.

Fratti, R.A., Jun, Y., Merz, A.J., Margolis, N., and Wickner, W. (2004) Interdependent assembly of specific regulatory lipids and membrane fusion proteins into the vertex ring domain of docked vacuoles. *J Cell Biol* **167**: 1087-1098.

Freeman, J.A., Ohl, M.E., and Miller, S.I. (2003) The *Salmonella* enterica Serovar Typhimurium Translocated Effectors SseJ and SifB Are Targeted to the *Salmonella*-Containing Vacuole. *Infect Immun* **71**: 418-427.

Freeman, J.A., Rappl, C., Kuhle, V., Hensel, M., and Miller, S.I. (2002) SpiC Is Required for Translocation of *Salmonella* Pathogenicity Island 2 Effectors and Secretion of Translocon Proteins SseB and SseC. *Journal of Bacteriology* **184**: 4971-4980.

Friebel, A., Ilchmann, H., Aepfelbacher, M., Ehrbar, K., Machleidt, W., and Hardt, W.D. (2001) SopE and SopE2 from *Salmonella* typhimurium activate different sets of RhoGTPases of the host cell. *J Biol Chem* **276**: 34035-34040.

Funderburk, S.F., Wang, Q.J., and Yue, Z. (2010) The Beclin 1-VPS34 complex--at the crossroads of autophagy and beyond. *Trends Cell Biol* **20**: 355-362.

Galan, J.E. (2009) Common themes in the design and function of bacterial effectors. *Cell Host Microbe* **5**: 571-579.

Galán, J.E. (2001) *Salmonella* Interactions with Host Cells: Type III Secretion at Work. *Annu Rev Cell Dev Biol* **17**: 53-86.

Galan, J.E., and Wolf-Watz, H. (2006) Protein delivery into eukaryotic cells by type III secretion machines. *Nature* **444**: 567-573.

Gal-Mor, O., Boyle, E.C., and Grassl, G.A. (2014) Same species, different diseases: how and why typhoidal and non-typhoidal *Salmonella* enterica serovars differ. *Front Microbiol* **5**: 391.

Garcia-Bustos, J.F., Marini, F., Stevenson, I., Frei, C., and and Hall, M.N. (1994) Pik1, an essential phosphatidylinositol 4-kinase associated with the yeast nucleus. *EMBO J* **10**: 2352-2361.

Garmendia, J., Beuzon, C.R., Ruiz-Albert, J., and Holden, D.W. (2003) The roles of SsrA-SsrB and OmpR-EnvZ in the regulation of genes encoding the *Salmonella* typhimurium SPI-2 type III secretion system. *Microbiology* **149**: 2385-2396.

Gary, J.D., Wurmser, A.E., Bonangelino, C.J., Weisman, L.S., and Emr, S.D. (1998) Fab1p Is Essential for PtdIns(3)P 5-Kinase Activity and the Maintenance of Vacuolar Size and Membrane Homeostasi. *J Cell Biol* **143**: 65–79.

Gaspar, A.H., and Machner, M.P. (2014) VipD is a Rab5-activated phospholipase A1 that protects *Legionella pneumophila* from endosomal fusion. *PNAS* **111**: 4560–4565.

Geddes, K., Worley, M., Niemann, G., and Heffron, F. (2005) Identification of new secreted effectors in *Salmonella* enterica serovar Typhimurium. *Infect Immun* **73**: 6260-6271

Gendrin, C., Contreras-Martel, C., Bouillot, S., Elsen, S., Lemaire, D., Skoufias, D.A., Huber, P., Attree, I., and Dessen, A. (2012) Structural basis of cytotoxicity mediated by the type III secretion toxin ExoU from Pseudomonas aeruginosa. *PLoS Pathog* **8**.

Ghaemmaghami, S., Huh, W.K., Bower, K., Howson, R.W., Belle, A., Dephoure, N., O'Shea, E.k., and and Weissman, J.S. (2003) Global analysis of protein expression in yeast. *Nature* **427**: 737-741.

Giacomodonato, M.N., Uzzau, S., Bacciu, D., Caccuri, R., Sarnacki, S.H., Rubino, S., and Cerquetti, M.C. (2007) SipA, SopA, SopB, SopD and SopE2 effector proteins of *Salmonella* enterica serovar Typhimurium are synthesized at late stages of infection in mice. *Microbiology* **153**: 1221-1228.

Grimm, S. (2012) The ER-mitochondria interface: the social network of cell death. *Biochim Biophys Acta* **1823**: 327-334.

Groisman, E.A., and Mouslim, C. (2000) Molecular mechanisms of *Salmonella* pathogenesis. *Curr Opin Infect Dis* **13**: 519-522.

Giaever, G., Chu, A. M., LiNi, Carla Connelly, Linda Riles, Steeve Ve´ronneau, Sally Dow, Ankuta Lucau-Danila, Keith Anderson,, Bruno Andre´, A.P.A., Anna Astromoff, Mohamed El Bakkoury, Rhonda Bangham, Rocio Benito, Sophie Brachat,, Stefano Campanaro, M.C., Karen Davis, Adam Deutschbauer, Karl-Dieter Entian, Patrick Flaherty, Francoise Foury,, David J. Garfinkel, M.G., Deanna Gotte, Ulrich Gu¨ldener, Johannes H. Hegemann, Svenja Hempel, Zelek Herman,, Daniel F. Jaramillo, D.E.K., Steven L. Kelly, Peter Ko¨ tter, Darlene LaBonte, David C. Lamb, Ning Lan, Hong Liang,, Hong Liao, L.L., Chuanyun Luo, Marc Lussier, Rong Mao, Patrice Menard, Siew Loon Ooi, Jose L. Revuelta,, Christopher J. Roberts, M.R., Petra Ross-Macdonald, Bart Scherens, Greg Schimmack, Brenda Shafer,, Daniel D. Shoemaker, S.S.-M., Reginald K. Storms, Jeffrey N. Strathern, Giorgio Valle, Marleen Voet,, Guido Volckaert, C.-y.W., Teresa R. Ward, Julie Wilhelmy, Elizabeth . Winzeler, Yonghong Yang, Grace Yen,, and Elaine Youngman, K.Y., Howard Bussey, Jef D. Boeke, Michael Snyder, Peter Philippsen, Ronald W. Davis & Mark Johnston (2002) Functional profiling of the Saccharomyces cerevisiae genome. *Nature* **418**: 387-391.

Guiney, D.G., and Fierer, J. (2011) The Role of the spv Genes in *Salmonella* Pathogenesis. *Front Microbiol* **2**: 129.

Günster, R.A., Matthews, S.A., Holden, D.W., and Thurston, T.L. (2017) SseK1 and SseK3 Type III Secretion System Effectors Inhibit NF-kappaB Signaling and Necroptotic Cell Death in Salmonella-Infected Macrophages. *Infect Immun* **85**.

Gurunathan, S., David, D., and Gerst, J.E. (2002) Dynamin and clathrin are required for the biogenesis of a distinct class of secretory vesicles in yeast. *EMBO J* **21**: 602-614.

Hallstrom, K.N., and McCormick, B.A. (2016) The type three secreted effector SipC regulates the trafficking of PERP during *Salmonella* infection. *Gut Microbes* **7**: 136-145.

Ham, H., Sreelatha, A., and Orth, K. (2011) Manipulation of host membranes by bacterial effectors. *Nat Rev Microbiol* **9**: 635-646.

Hama, H., Schnieders, E.A., Thorner, J., Takemoto, J.Y., and and DeWald, D.B. (1999) Direct Involvement of Phosphatidylinositol 4-Phosphate in Secretion in the Yeast Saccharomyces cerevisiae. *The Journal of Biological Chemistry* **274**: 34294–34300.

Han, G.S., Audhya, A., Markley, D.J., Emr, S.D., and Carman, G.M. (2002) The Saccharomyces cerevisiae LSB6 gene encodes phosphatidylinositol 4-kinase activity. *J Biol Chem* **277**: 47709-47718.

Hanaha, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol 166*, 557-580.

Haneda, T., Ishii, Y., Shimizu, H., Ohshima, K., Iida, N., Danbara, H., and Okada, N. (2012) *Salmonella* type III effector SpvC, a phosphothreonine lyase, contributes to reduction in inflammatory response during intestinal phase of infection. *Cell Microbiol* **14**: 485-499.

Haraga, A., Ohlson, M.B., and Miller, S.I. (2008) *Salmonellae* interplay with host cells. *Nat Rev Microbio* **6**: 53-66.

Hartwell, L.H. (2002) Yeast and cancer. *Bioscience reports* **22**: 373-394.

Hayward, R.D., McGhie, E.J., and Koronakis, V. (2000) Membrane fusion activity of purified SipB, a Salmonella surface protein essential for mammalian cell invasion. *Molecular Microbiology* **37**: 727-739.

He, B., Xi, F., Zhang, X., Zhang, J., and Guo, W. (2007) Exo70 interacts with phospholipids and mediates the targeting of the exocyst to the plasma membrane. *EMBO J* **26**: 4053–4065.

Hersh, D., Monack, D.M., Smith, M.R., Ghori, N.G., Falkow, S.F., and Zychlinsky, A. (1999) The Salmonella invasin SipB induces macrophage apoptosis by binding to caspase-1.*Microbiol* **96**: 2396–2401.

Henne, W.M., Buchkovich, N.J., and Emr, S.D. (2011) The ESCRT pathway. *Dev Cell* **21**: 77-91.

Henry, T., Couillault, C., Rockenfeller, P., Boucrot, E., Dumont, A., Schroeder, N., Hermant, A., Knodler, L.A., Lecine, P., Steele-Mortimer, O., Borg, J.P., Gorvel, J.P., and Meresse, S. (2006) The *Salmonella* effector protein PipB2 is a linker for kinesin-1. *Proc Natl Acad Sci U S A* **103**: 13497-13502.

Hensel, M. (2004) Evolution of pathogenicity islands of *Salmonella* enterica. *Int J Med Microbiol* **294**: 95-102.

Heyndrickx, M., Pasmans, F., Ducatelle, R., Decostere, A., and Haesebrouck, F. (2005) Recent changes in *Salmonella* nomenclature: the need for clarification. *Vet J* **170**: 275-277.

Hicks, S.W., Charron, G., Hang, H.C., and Galan, J.E. (2011) Subcellular targeting of *Salmonella* virulence proteins by host-mediated S-palmitoylation. *Cell Host Microbe* **10**: 9-20.

Hicks, S.W., and Galan, J.E. (2013) Exploitation of eukaryotic subcellular targeting mechanisms by bacterial effectors. *Nat Rev Microbiol* **11**: 316-326.

Hirst, J., Lui, W.W.Y., Bright, N.A., Totty, N., Seaman, M.N.J., and and Robinson, M.S. (2000) A Family of Proteins with γ -Adaptin and VHS Domains that Facilitate Trafficking between the Trans-Golgi Network and the Vacuole/Lysosome. *The Journal of Cell Biology* **149**: 67-79.

Ho, C.Y., Alghamdi, T.A., and Botelho, R.J. (2012) Phosphatidylinositol-3,5-bisphosphate: no longer the poor PIP2. *Traffic* **13**: 1-8.

Hochmann, H., Pust, S., von Figura, G., Aktories, K., and Barth, H. (2006) *Salmonella* enterica SpvB ADP-Ribosylates Actin at Position Arginine-177s Characterization of the Catalytic Domain within the SpvB Protein and a Comparison to Binary Clostridial Actin-ADP-Ribosylating Toxins. *Biochemistry* **45**: 1271-1277.

Horazdovsky, B.F., Davies, B.A. Seaman, M.N.J., McLaughlin, S.A., Yoon, S-H., and Emr,S.D. (1997) A Sorting Nexin-1 Homologue, Vps5p, Forms a Complex with Vpsl7p and Is Required for Recycling the Vacuolar Protein-sorting Receptor. *Molecular Biology of the Cell* **8**: 1529-1541.

Hubber, A., Arasaki, K., Nakatsu, F., Hardiman, C., Lambright, D., De Camilli, P., Nagai, H., and Roy, C.R. (2014) The machinery at endoplasmic reticulum-plasma membrane contact sites contributes to spatial regulation of multiple Legionella effector proteins. *PLoS Pathog* **10**: e1004222.

Hughes, W.E., Cooke, F.T., and Parker, P.J. (2000) Sac phosphatase domain proteins. *Biochem J* **350**: 337–352.

Huh, W.K., Falvo, J.V., Gerke, L.C., Carroll, A.S., Howson, R.W., Weissman, J.S., and and O'Shea, E.K. (2003) Global analysis of protein localization in budding yeast. *Nature* **425**: 686-691.

Humphreys, D., Davidson, A., Hume, P.J., and Koronakis, V. (2012) *Salmonella* virulence effector SopE and Host GEF ARNO cooperate to recruit and activate

WAVE to trigger bacterial invasion. *Cell Host Microbe* **11**: 129-139.

Humphreys, D., Hume, P.J., and Koronakis, V. (2009) The *Salmonella* effector SptP dephosphorylates host AAA+ ATPase VCP to promote development of its intracellular replicative niche. *Cell Host Microbe* **5**: 225-233.

Hurley, D., McCusker, M.P., Fanning, S., and Martins, M. (2014) *Salmonella*-host interactions - modulation of the host innate immune system. *Front Immunol* **5**: 481.

Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983). Transformation of intact yeast cells treated with álcali cations. *J Bacteriol* **153**: 163-168.

Imke hansen-wester, M.h. (2001) *Salmonella* pathogenicity islands encoding type III secretion systems. *Microb Infect* **3**: 549-559.

Jain, P., Luo, Z.K., and Blanke, S.R. (2011) Helicobacter pylori vacuolating cytotoxin A (VacA) engages the mitochondrial fission machinery to induce host cell death. *PNAS* **108**: 16032-16037.

Jank, T., Bohmer, K.E., Tzivelekidis, T., Schwan, C., Belyi, Y., and Aktories, K. (2012) Domain organization of Legionella effector SetA. *Cell Microbiol* **14**: 852-868.

Jepson, M.A., Kenny, B., and and Leard, A.D. (2001) Role of sipA in the early stages of *Salmonella* Typhimurium entry into epithelial cells. *Cell Microbiol* **3**: 417-426.

Jiang, X., Rossanese, O.W., Brown, N.F., Kujat-Choy, S., Galan, J.E., Finlay, B.B., and Brumell, J.H. (2004) The related effector proteins SopD and SopD2 from *Salmonella* enterica serovar Typhimurium contribute to virulence during systemic infection of mice. *Mol Microbiol* **54**: 1186-1198.

Jin , N., Chow, C.Y., Liu , L., Zolov, S.N., Bronson, R., Davisson, M., Petersen, J.L., Zhang, Y., Park, S., Duex, J.E., Goldowitz, D., Meisler, M.H., and and Weisman, L.S. (2008) VAC14 nucleates a protein complex essential for the acute interconversion of PI3P and PI(3,5)P2 in yeast and mouse. *EMBO J* **27**: 3221–3234.

Johnson, C.M., and Rodgers, W. (2008) Spatial Segregation of Phosphatidylinositol 4,5-Bisphosphate (PIP(2)) Signaling in Immune Cell Functions. *Immunol Endocr Metab Agents Med Chem* **8**: 349-357.

Kashiwazaki, J., Iwaki, T., Takegawa, K., Shimoda, C., and Nakamura, T. (2009) Two fission yeast rab7 homologs, ypt7 and ypt71, play antagonistic roles in the regulation of vacuolar morphology. *Traffic* **10**: 912-924.

Kachroo, A.H., Laurent, J.M., Yellman, C.M., Meyer, A.G., Wilke, C.O., and Marcotte, E.M. (2015) Systematic humanization of yeast genes reveals conserved functions and genetic modularity. *Science* **348**: 921-925.

Keune, W., Bultsma, Y., Sommer, L., Jones, D., and Divecha, N. (2011) Phosphoinositide signalling in the nucleus. *Adv Enzyme Regul* **51**: 91-99.

Kimbrough, T.G., and Miller, S.I. (2000) Contribution of *Salmonella* typhimurium type III secretion components to needle complex formation. *Proc Natl Acad Sci U S A* **97**: 11008-11013.

Knodler, L.A., Finlay, B.B., and Steele-Mortimer, O. (2005) The *Salmonella* effector protein SopB protects epithelial cells from apoptosis by sustained activation of Akt. *J Biol Chem* **280**: 9058-9064.

Knodler, L.A., and Steele-Mortimer, O. (2005) The *Salmonella* effector PipB2 affects late endosome/lysosome distribution to mediate Sif extension. *Mol Biol Cell* **16**: 4108-4123.

Knodler, L.A., Vallance, B.A., Hensel, M., Jäckel, D., Finlay, B.B., and Steele-Mortimer, O. (2004) *Salmonella* type III effectors PipB and PipB2 are targeted to detergent-resistant microdomains on internal host cell membranes. *Mol Microbiol* **49**: 685-704.

Kolodziejek, A.M., and Miller, S.I. (2015) *Salmonella* modulation of the phagosome membrane, role of SseJ. *Cell Microbiol* **17**: 333-341.

Kornmann , B., and Walter, P. (2010) ERMES-mediated ER-mitochondria contacts: molecular hubs for the regulation of mitochondrial biology. *J Cell Sci* **123**: 1389-1393.

Kuhle, V., and Hensel, M. (2004) Cellular microbiology of intracellular *Salmonella* enterica: functions of the type III secretion system encoded by *Salmonella* pathogenicity island 2. *Cell Mol Life Sci* **61**: 2812-2826.

Kujat Choy, S.L., Boyle, E.C., Gal-Mor, O., Goode, D.L., Valdez, Y., Vallance, B.A., and Finlay, B.B. (2004) SseK1 and SseK2 are novel translocated proteins of *Salmonella* enterica serovar typhimurium. *Infect Immun* **72**: 5115-5125.

Kutateladze, T.G. (2010) Translation of the phosphoinositide code by PI effectors. *Nat Chem Biol* **6**: 507-513.

Lara-Tejero, M., and Galan, J.E. (2009) *Salmonella* enterica serovar typhimurium pathogenicity island 1-encoded type III secretion system translocases mediate intimate attachment to nonphagocytic cells. *Infect Immun* **77**: 2635-2642.

LaRock, D.L., Chaudhary, A., and Miller, S.I. (2015) *Salmonella*e interactions with host processes. *Nat Rev Microbiol* **13**: 191-205.

Lauwers, E., Erpapazoglou, Z., Haguenauer-Tsapis, R., and Andre, B. (2010) The ubiquitin code of yeast permease trafficking. *Trends Cell Biol* **20**: 196-204.

Lawley, T.D., Chan, K., Thompson, L.J., Kim, C.C., Govoni, G.R., and Monack, D.M. (2006) Genome-wide screen for *Salmonella* genes required for long-term systemic infection of the mouse. *PLoS Pathog* **2**: e11.

Layton, A.N., Brown, P.J., and Galyov, E.E. (2005) The *Salmonella* translocated effector SopA is targeted to the mitochondria of infected cells. *J Bacteriol* **187**: 3565-3571.

Lebreton, A., Stavru, F., and Cossart, P. (2015) Organelle targeting during bacterial infection: insights from Listeria. *Trends Cell Biol* **25**: 330-338.

Lesnick, M.L., Reiner, N.E., Fierer, J., and Guiney, D.G. (2001) The *Salmonella* spvB virulence gene encodes an enzyme that ADP-ribosylates actin and destabilizes the cytoskeleton of eukaryotic cells. *Mol Microbiol* **39**: 1464-1470.

Lesser, C.F., and and Miller, S.I. (2001) Expression of microbial virulence proteins in Saccharomyces cerevisiae models mammalian infection. *EMBO J* **20**: 1840-1849.

Li, D., Wang, X., Wang, L., and Zhoub, D. (2013) The Actin-Polymerizing Activity of SipA Is Not Essential for *Salmonella enterica* Serovar Typhimurium-Induced Mucosal Inflammation. *Infect Immun* **81**: 1541-1549.

Li, H., Xu, H., Zhou, Y., Zhang, I., Long, C., Li, S., Chen, S., Zhou, J.M., and F., a.S. (2007) The phosphothreonine lyase activity of a bacterial type III effector family. *Science* **315**: 1000-1003.

Liao, A.P., Petrof, E.O., Kuppireddi, S., Zhao, Y., Xia, Y., Claud, E.C., and Sun, J. (2008) *Salmonella* type III effector AvrA stabilizes cell tight junctions to inhibit inflammation in intestinal epithelial cells. *PLoS One* **3**: e2369.

Lilic, M., Galkin, V.E., Orlova, A., VanLoock, M.S., Egelman, E.H., and & Stebbins, C.E. (2003) *Salmonella* SipA Polymerizes Actin by Stapling Filaments with Nonglobular Protein Arms. *Science* **301**: 1918-1921.

Lin, S.L., Le, T.X., and Cowen, D.S. (2003) SptP, a *Salmonella* typhimurium type III-secreted protein, inhibits the mitogen-activated protein kinase pathway by inhibiting Raf activation. *Cellular Microbiology* **5**: 267-275.

Ling, Y., Hayano, S., and Novick, P. (2014) Osh4p is needed to reduce the level of phosphatidylinositol-4-phosphate on secretory vesicles as they mature. *Molecular Biology of the Cell* **25**: 3389-3400.

Liua, T.T., Gomezb, T.S., Sackeya, B.K., Billadeaub, B.D., and and Burda, C.G. (2012) Rab GTPase regulation of retromer-mediated cargo export during endosome maturation. *Mol Biol Cell* **23**: 2505-2515.

Lorente-Rodríguez, A., and and Barlowe, C. (2011) Requirement for Golgi-localized PI(4)P in fusion of COPII vesicles with Golgi compartments. *Molr Biol Cell* **22**: 216-229.

Lossi, N.S., Rolhion, N., Magee, A.I., Boyle, C., and Holden, D.W. (2008) The *Salmonella* SPI-2 effector SseJ exhibits eukaryotic activator-dependent phospholipase A and glycerophospholipid: cholesterol acyltransferase activity. *Microbiology* **154**: 2680-2688.

Luo, X., Wasilko, D.J., Liu, Y., Sun, J., Wu, X., Luo, Z.Q., and Mao, Y. (2015) Structure of the Legionella Virulence Factor, SidC Reveals a Unique PI(4)P-Specific Binding Domain Essential for Its Targeting to the Bacterial Phagosome. *PLoS Pathog* **11**: e1004965.

Madeo, F., Engelhardt, S., Herker, E., Lehmann, N., Maldener, C., Proksch, A., Wissing, S., and Frohlich, K.U. (2002) Apoptosis in yeast: a new model system with applications in cell biology and medicine. *Curr Genet* **41**: 208-216.

Madeo, F., Fröhlich, E., and and Fröhlich, K.W. (2007) A Yeast Mutant Showing Diagnostic Markers of Early and Late Apoptosis. *J Cell Biol* **139**: 729–734.

Malik-Kale, P., Jolly, C.E., Lathrop, S., Winfree, S., Luterbach, C., and Steele-Mortimer, O. (2011) *Salmonella* - at home in the host cell. *Front Microbiol* **2**: 125.

Manik, M.K., Yang, H., Tong, J., and Im, Y.J. (2017) Structure of Yeast OSBP-Related Protein Osh1 Reveals Key Determinants for Lipid Transport and Protein Targeting at the Nucleus-Vacuole Junction. *Structure*.

Mao, X., Hu, Y., Liang, C., and Lu, C. (2002) MET3 promoter: a tightly regulated promoter and its application in construction of conditional lethal strain. *Curr Microbiol* **45**: 37-40.

Marat, A.L., and Haucke, V. (2016) Phosphatidylinositol 3-phosphates-at the interface between cell signalling and membrane traffic. *EMBO J* **35**: 561-579.

Marchi, S., Patergnani, S., and Pinton, P. (2014) The endoplasmic reticulum-mitochondria connection: one touch, multiple functions. *Biochim Biophys Acta* **1837**: 461-469.

Marcus, S.L., Brumell, J.H., Pfeifer, C.G., and & Finlay, B.B. (2000) *Salmonella* pathogenicity islands: big virulence in small packages. *Microb Infect* **2**: 145-156.

Marcusson, E.G., Cereghino, J.L., Horazdovsky, B.F., Gharakhanian, E., and and Emr, S.D. (1994) The Sorting Receptor for Yeast Vacuolar Carboxypeptidase Y Is Encoded by the VFW0 Gene. *Cell* **77**: 579-596.

Markgraf, D.F., Ahnert, F., Arlt, H., Mari, M., Peplowska, K., Epp, N., Griffith, J., Reggiori, F., and and Ungermann, C. (2009) The CORVET Subunit Vps8 Cooperates with the Rab5 Homolog Vps21 to Induce Clustering of Late Endosomal Compartments. *Mol Biol Cell* **20**: 5276–5289.

Marcus, S.L., and Finlay, B.B. (2002) Salmonella enterica serovar Typhimurium effector SigD/SopB is membrane-associated and ubiquitinated inside host cells. *Cell Microbiol* **4**: 435–446.

Mayer, A., Wickner, W., Scheglmann, D., and Haas, A. (2000) Phosphatidylinositol 4,5-bisphosphate regulates Two steps of homotypic vacuole fusion. *Mol Biol Cell* **11**: 807-817.

Mayinger, P. (2012) Phosphoinositides and vesicular membrane traffic. *Biochim Biophys Acta* **1821**: 1104-1113.

Mazurkiewicz, P., Thomas, J., Thompson, J.A., Liu, M., Arbibe, L., Sansonetti, P., and Holden, D.W. (2008) SpvC is a *Salmonella* effector with phosphothreonine lyase activity on host mitogen-activated protein kinases. *Mol Microbiol* **67**: 1371-1383.

McClelland, M., Sanderson, K.E., Spieth, J., Clifton, S.W., Latreille, P., Courtney, L., Porwollik, Ali, J., Dante, M., Du, F., Hou, S., Layman, D., Leonard, S., Nguyen, C., Scott, K., Holmes, A., Grewal, N., Mulvaney, E., Ryan, E., Sun, H., Florea, L., Miller, W., Stoneking, T., Nhan, M., Waterston, R., and & Wilson, R.K. (2001) Complete genome sequence of *Salmonella* enterica serovar Typhimurium LT2. *Nature* **413**: 852-856.

McGhie, E.J., Brawn, L.C., Hume, P.J., Humphreys, D., and Koronakis, V. (2009) *Salmonella* takes control: effector-driven manipulation of the host. *Curr Opin Microbiol* **12**: 117-124.

McGhie, E.J., Hayward, R.D., and and Koronakis, V. (2001) Cooperation between actin-binding proteins of invasive *Salmonella*: SipA potentiates SipC nucleation and bundling of actin. *EMBO J* **20**: 2131-2139.

McLaughlin, L.M., Govoni, G.R., Gerke, C., Gopinath, S., Peng, K., Laidlaw, G., Chien, Y.H., Jeong, H.W., Li, Z., Brown, M.D., Sacks, D.B., and Monack, D. (2009) The *Salmonella* SPI2 effector SseI mediates long-term systemic infection by modulating host cell migration. *PLoS Pathog* **5**

McLaughlin, S., and Murray, D. (2005) Plasma membrane phosphoinositide organization by protein electrostatics. *Nature* **438**: 605-611.

Mesquita, F.S., Thomas, M., Sachse, M., Santos, A.J., Figueira, R., and Holden, D.W. (2012) The *Salmonella* deubiquitinase SseL inhibits selective autophagy of cytosolic aggregates. *PLoS Pathog* **8**: e1002743.

Miao, E.A., Brittnacher, M., Haraga, A., Jeng, R.L., Welch, M.D., and Miller, S.I. (2003) *Salmonella* effectors translocated across the vacuolar membrane interact with the actin cytoskeleton. *Mol Microbiol* **48**: 401-415.

Michel, A.H., and Kornmann, B. (2012) The ERMES complex and ER-mitochondria connections. *Biochem Soc Trans* **40**: 445-450.

Mitchell, D.A., Marshall, T.K., and & Deschenes, R.J. (1993) Vectors for the Inducible Overexpression of Glutathione S-Transferase Fusion Proteins in Yeast. *Yeast* **9**: 713-725.

Molero, C., Rodríguez-Escudero, I., Aleman, A., Rotger, R., Molina, M., and Cid, V.J. (2009) Addressing the effects of *Salmonella* internalization in host cell signaling on a reverse-phase protein array. *Proteomics* **9**: 3652-3665

Outeiro, T.F., and Lindquist, S. (2003) Yeast Cells Provide Insight into Alpha-Synuclein Biology and Pathobiology. *Science* **502**: 1772–1775.

Overington, J.P., Al-Lazikani, B., and Hopkins, A.L. (2006) How many drug targets are there? *Nat rev* **5**: 993-996.

Von Filseck, J., Vanni, S., Mesmin, B., Antonny, B., and Drin, G. (2015) A phosphatidylinositol-4-phosphate powered exchange mechanism to create a lipid gradient between membranes. *Nat Commun* **6**: 6671.

Mima, J., and Wickner, W. (2009) Phosphoinositides and SNARE chaperones synergistically assemble and remodel SNARE complexes for membrane fusion. *Proc Natl Acad Sci U S A* **106**: 16191-16196.

Muller, P., Chikkaballi, D., and Hensel, M. (2012) Functional dissection of SseF, a membrane-integral effector protein of intracellular *Salmonella* enterica. *PLoS One* **7**: e35004.

Myeni, S.K., and Zhou, D. (2010) The C terminus of SipC binds and bundles F-actin to promote *Salmonella* invasion. *J Biol Chem* **285**: 13357-13363.

Nejedlik, L., Pierfelice, T., and Geiser, J.R. (2004) Actin distribution is disrupted upon expression of Yersinia YopO/YpkA in yeast. *Yeast* **21**: 759-768.

Nelson, J., Gibbons, E., Pickett, K.R., Streeter, M., Warcup, A.O., Yeung, C.H., Judd, A.M., and Bell, J.D. (2011) Relationship between membrane permeability and specificity of human secretory phospholipase A(2) isoforms during cell death. *Biochim Biophys Acta* **1808**: 1913-1920

Niemann, G.S., Brown, R.N., Gustin, J.K., Stufkens, A., Shaikh-Kidwai, A.S., Li, J., McDermott, J.E., Brewer, H.M., Schepmoes, A., Smith, R.D., Adkins, J.N., and Heffron, F. (2011) Discovery of novel secreted virulence factors from *Salmonella* enterica serovar Typhimurium by proteomic analysis of culture supernatants. *Infect Immun* **79**: 33-43.

Obara, K., Noda, T., Niimi, K., and Ohsumi, Y. (2008) Transport of phosphatidylinositol 3-phosphate into the vacuole via autophagic membranes in *Saccharomyces cerevisiae*. *Genes Cells* **13**: 537-547.

Obara, K., and Ohsumi, Y. (2011) PtdIns 3-Kinase Orchestrates Autophagosome Formation in Yeast. *J Lipids* **2011**: 498768.

O'Brien, K.M., Lindsay, E.L., and Starai, V.J. (2015) The *Legionella pneumophila* effector protein, LegC7, alters yeast endosomal trafficking. *PLoS One* **10**: e0116824.

Odendall, C., Rolhion, N., Forster, A., Poh, J., Lamont, D.J., Liu, M., Freemont, P.S., Catling, A.D., and Holden, D.W. (2012) The *Salmonella* kinase SteC targets the MAP kinase MEK to regulate the host actin cytoskeleton. *Cell Host Microbe* **12**: 657-668.

Odorizzi, G., Babst, M., and Emr, S.D. (2000) Phosphoinositide signaling and the regulation of membrane trafficking in yeast. *TIBS* **25**: 229-235.

Odorizzi, G., Cowles, C.R., and Emr, S.D. (1998) The AP-3 complex: a coat of many colours. *Tren Cell Biol* **8**: 282–288.

Otto, H., Tezcan-Merdol, D., Girisch, R., Haag, F., Rhen, M., and and Koch-Nolte, F. (2000) The spvB gene-product of the Salmonella enterica virulence plasmid is a mono(ADP-ribosyl)transferase. *Cell Microbiol* **37(5)**: 1106-1115.

Patel, J.C., Hueffer, K., Lam, T.T., and Galan, J.E. (2009) Diversification of a

Salmonella virulence protein function by ubiquitin-dependent differential localization. *Cell* **137**: 283-294.

Patel, J.C., and Galan, J.E. (2005) Manipulation of the host actin cytoskeleton by Salmonella--all in the name of entry. *Curr Opin Microbiol* **8**: 10-15.

Pausch, M.H. (1997) G-protein-coupled receptors in Saccharomyces cerevisiae: "high-throughput screening assays for drug discovery". *Trends Biotechnol* **15**: 487-494.

Pelham, M.W.B.a.H.R.B. (2000) A Selective Transport Route from Golgi to Late Endosomes that Requires the Yeast GGA Proteins *J Cell Biol* **151**: 587-600

Peng, B., C., T., Williams, M., Henry, L., Nielsen, K., and Vickers, C.E. (2015) Controlling heterologous gene expression in yeast cell factories on different carbon substrates and across the diauxic shift: a comparison of yeast promoter activities. *Microbial cell factories* **14**.

Peplowska, K., Markgraf, D.F., Ostrowicz, C.W., Bange, G., and Ungermann, C. (2007) The CORVET tethering complex interacts with the yeast Rab5 homolog Vps21 and is involved in endo-lysosomal biogenesis. *Dev Cell* **12**: 739-750.

Perrett, C.A., and Zhou, D. (2013) *Salmonella* type III effector SopB modulates host cell exocytosis. *Emerg Microbes Infect* **2**: e32.

Perrone, G.G., Tan, S.X., and Dawes, I.W. (2008) Reactive oxygen species and yeast apoptosis. *Biochim Biophys Acta* **1783**: 1354-1368.

Phan, H.L., Finlay, J.A., Chu, D.S., Tan, P.K., Kirchhausen, T., and and Payne, G.S. (1994) The Saccharomyces cerevisiae APS1 gene encodes a homolog of the small subunit of the mammalian clathrin AP-1 complex: evidence for functional interaction with clathrin at the Golgi complex. *EMBO J* **13**: 1706-1717.

Pilar, A.V., Reid-Yu, S.A., Cooper, C.A., Mulder, D.T., and Coombes, B.K. (2012) GogB is an anti-inflammatory effector that limits tissue damage during *Salmonella* infection through interaction with human FBXO22 and Skp1. *PLoS Pathog* **8**: e1002773.

Poh, J., Odendall, C., Spanos, A., Boyle, C., Liu, M., Freemont, P., and Holden, D.W. (2008) SteC is a *Salmonella* kinase required for SPI-2-dependent F-actin remodelling. *Cell Microbiol* **10**: 20-30.

Popa, C., Coll, N.S., Valls, M., and Sessa, G. (2016) Yeast as a Heterologous Model System to Uncover Type III Effector Function. *PLoS Pathog* **12**: e1005360.

Que, F., Wu, S., and Huang, R. (2013) *Salmonella* pathogenicity island 1(SPI-1) at work. *Curr Microbiol* **66**: 582-587.

Raffatellu, M., Wilson, R.P., Chessa, D., Andrews-Polymenis, H., Tran, Q.T., Lawhon, S., Khare, S., Adams, L.G., and Baumler, A.J. (2005) SipA, SopA, SopB, SopD, and SopE2 contribute to *Salmonella* enterica serotype typhimurium invasion of epithelial cells. *Infect Immun* **73**: 146-154.

Ragaz, C., Pietsch, H., Urwyler, S., Tiaden, A., Weber, S.S., and Hilbi, H. (2008) The Legionella pneumophila phosphatidylinositol-4 phosphate-binding type IV substrate SidC recruits endoplasmic reticulum vesicles to a replication-permissive vacuole. *Cell Microbiol* **10**: 2416-2433.

Ramos-Morales, F. (2012) Impact of *Salmonella* enterica Type III Secretion System Effectors on the Eukaryotic Host Cell. *ISRN Cell Biology* **2012**: 1-36.

Ramsden, A.E., Holden, D.W., and Mota, L.J. (2007) Membrane dynamics and spatial distribution of *Salmonella*-containing vacuoles. *Trends Microbiol* **15**: 516-524.

Raymond, B., Young, J.C., Pallett, M., Endres, R.G., Clements, A., and Frankel, G. (2013) Subversion of trafficking, apoptosis, and innate immunity by type III secretion system effectors. *Trends Microbiol* **21**: 430-441.

Reinicke, A.T., Hutchinson, J.L., Magee, A.I., Mastroeni, P., Trowsdale, J., and Kelly, A.P. (2005) A *Salmonella* typhimurium effector protein SifA is modified by host cell prenylation and S-acylation machinery. *J Biol Chem* **280**: 14620-14627.

Rodríguez-Escudero, I., Andres-Pons, A., Pulido, R., Molina, M., and Cid, V.J. (2009) Phosphatidylinositol 3-kinase-dependent activation of mammalian protein kinase B/Akt in Saccharomyces cerevisiae, an in vivo model for the functional study of Akt mutations. *J Biol Chem* **284**: 13373-13383.

Rodríguez-Escudero, I., Ferrer, N.L., Rotger, R., Cid, V.J., and Molina, M. (2011) Interaction of the *Salmonella* Typhimurium effector protein SopB with host cell Cdc42 is involved in intracellular replication. *Mol Microbiol* **80**: 1220-1240.

Rodríguez-Escudero, I., Hardwidge, P.R., Nombela, C., Cid, V.J., Finlay, B.B., and Molina, M. (2005) Enteropathogenic Escherichia coli type III effectors alter cytoskeletal function and signalling in Saccharomyces cerevisiae. *Microbiology* **151**: 2933-2945.

Rodríguez-Escudero, I., Rotger, R., Cid, V.J., and Molina, M. (2006) Inhibition of Cdc42-dependent signalling in Saccharomyces cerevisiae by phosphatase-dead SigD/SopB from *Salmonella* typhimurium. *Microbiology* **152**: 3437-3452.

Rodríguez-Pachón, J.M., Martin, H., North, G., Rotger, R., Nombela, C., and Molina, M. (2002) A novel connection between the yeast Cdc42 GTPase and the Slt2-mediated cell integrity pathway identified through the effect of secreted *Salmonella* GTPase modulators. *J Biol Chem* **277**: 27094-27102.

Rodríguez-Escudero, M., Cid, V.J., Molina, M., Schulze-Luehrmann, J., Luhrmann, A., and Rodríguez-Escudero, I. (2016) Studying Coxiella burnetii Type IV Substrates in the Yeast Saccharomyces cerevisiae: Focus on Subcellular Localization and Protein Aggregation. *PLoS One* **11**: e0148032.

Rohde, J.R., Breitkreutz, A., Chenal, A., Sansonetti, P.J., and Parsot, C. (2007) Type III secretion effectors of the IpaH family are E3 ubiquitin ligases. *Cell Host Microbe* **1**: 77-83

Rolhion, N., Furniss, R.C., Grabe, G., Ryan, A., Liu, M., Matthews, S.A., and Holden, D.W. (2016) Inhibition of Nuclear Transport of NF-kB p65 by the Salmonella Type III Secretion System Effector SpvD. *PLoS Pathog* **12**: e1005653.

Rostislavleva, K., Soler, N., Ohashi, Y., Zhang, L., Pardon, E., Burke, J.E., Masson, G.R., Johnson, C., Steyaert, J., Ktistakis, N.T., and Williams, R.L. (2015) Structure and flexibility of the endosomal Vps34 complex reveals the basis of its function on membranes. *Science* **350**: aac7365.

Roy, A., and Levine, T.P. (2004) Multiple pools of phosphatidylinositol 4-phosphate detected using the pleckstrin homology domain of Osh2p. *J Biol Chem* **279**: 44683-44689.

Rudge, S.A., Anderson, D.M., and Emr, S.D. (2004) Vacuole size control: regulation of PtdIns(3,5)P2 levels by the vacuole-associated Vac14-Fig4 complex, a PtdIns(3,5)P2-specific phosphatase. *Mol Biol Cell* **15**: 24-36.

Ruiz-Albert, J., Yu, X.J., Beuzón, C.R., Blakey, A.N., Galyov, E.E., and Holden, D.W. (2002) Complementary activities of SseJ and SifA regulate dynamics of the Salmonella Typhimurium vacuolar membrane. *Molecular Microbiology* **44(3)**: 645-661.

Rusten, T.E., Rodahl, L.M.W., Pattni, K., Englund, C., Samakovlis, C., Dove, S., Brech, A., and and Stenmark, H. (2006) Fab1 Phosphatidylinositol 3-Phosphate 5-Kinase Controls Trafficking but Not Silencing of Endocytosed Receptors. *Mol biol cell* **17**: 3989–4001.

Ryan, M., Graham, L.A., and and Stevens, T.H. (2008) Voa1p Functions in V-ATPase Assembly in the Yeast Endoplasmic Reticulum. *Mol Biol Cell* **19**: 5131–5142.

Rytkonen, A., Poh, J., Garmendia, J., Boyle, C., Thompson, A., Liu, M., Freemont, P., Hinton, J.C., and Holden, D.W. (2007) SseL, a *Salmonella* deubiquitinase required for macrophage killing and virulence. *Proc Natl Acad Sci U S A* **104**: 3502-3507.

Salomon, D., Bosis, E., Dar, D., Nachman, I., and Sessa, G. (2012) Expression of Pseudomonas syringae type III effectors in yeast under stress conditions reveals that HopX1 attenuates activation of the high osmolarity glycerol MAP kinase pathway. *Microbiology* **158**: 2859-2869.

Sambrook, S.L., Fritsch, E.F., and Maniatis, T. (1989). *Molecular cloning: a laboratory manual*. Cold spring Harbor Laboratory Press, New York.

Santiago-Tirado, F.H., and Bretscher, A. (2011) Membrane-trafficking sorting hubs: cooperation between PI4P and small GTPases at the trans-Golgi network. *Trends Cell Biol* **21**: 515-525.

Schimmöller, F., and and Riezman, H. (1993) Involvement of Ypt7p, a small GTPase, in traffic from late endosome to the vacuole in yeast. *J Cell Sci* **106**: 823-830.

Schlumberger, M.C., and Hardt, W.D. (2006) *Salmonella* type III secretion effectors: pulling the host cell's strings. *Curr Opin Microbiol* **9**: 46-54.

Schraidt, O., Lefebre, M.D., Brunner, M.J., Schmied, W.H., Schmidt, A., Radics, J., Mechtler, K., Galan, J.E., and Marlovits, T.C. (2010) Topology and organization of the *Salmonella* typhimurium type III secretion needle complex components. *PLoS Pathog* **6**: e1000824.

Schroeder, N., Henry, T., de Chastellier, C., Zhao, W., Guilhon, A.A., Gorvel, J.P., and Meresse, S. (2010) The virulence protein SopD2 regulates membrane dynamics of *Salmonella*-containing vacuoles. *PLoS Pathog* **6**: e1001002.

Schroeder, N., Mota, L.J., and Meresse, S. (2011) *Salmonella*-induced tubular networks. *Trends Microbiol* **19**: 268-277.

Seaman, M.N.J., Marcusson, E.G., Cereghino, J.L., and Emr,S.D. (1997) Endosome to Golgi Retrieval of the Vacuolar Protein Sorting Receptor, Vps10p, Requires the Function of the VPS29 , VPS30 , and VPS35 Gene Products. *J Cell Biol* **137**: 79–92.

Seaman, M.N.J., McCaffery, J.M., and Emr, S.D. (1998) A Membrane Coat Complex Essential for Endosome-to-Golgi Retrograde Transport in Yeast. *The Journal of Cell Biology* **142**: 665–681.

Sherman, F., Fink, G.R., and Hicks, J.B. (1986) *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, New York.

Shisheva, A. (2008) PIKfyve: Partners, significance, debates and paradoxes. *Cell Biol Int* **32**: 591-604.

Siggers, K.A., and Lesser, C.F. (2008) The Yeast Saccharomyces cerevisiae: a versatile model system for the identification and characterization of bacterial virulence proteins. *Cell Host Microbe* **4**: 8-15.

Simon, V. R., Swayne, T.C., and Pon, L.A. (1995) Actin-dependent. Mitochondrial motility in mitotic yeast and cell-free systems: identification of a motor activity on the mitochondrial surface. *Cell* **130**: 345–354.

Sisko, J.L., Spaeth, K., Kumar, Y., and Valdivia, R.H. (2006) Multifunctional analysis of Chlamydia-specific genes in a yeast expression system. *Mol Microbiol* **60**: 51-66.

Slagowski, N.L., Kramer, R.W., Morrison, M.F., LaBaer, J., and Lesser, C.F. (2008) A functional genomic yeast screen to identify pathogenic bacterial proteins. *PLoS Pathog* **4**: e9.

Sridhar, S., Patel, B., Aphkhazava, D., Macian, F., Santambrogio, L., Shields, D., and Cuervo, A.M. (2013) The lipid kinase PI4KIIIbeta preserves lysosomal identity. *EMBO J* **32**: 324-339.

Srikanth, C.V., Mercado-Lubo, R., Hallstrom, K., and McCormick, B.A. (2011) *Salmonella* effector proteins and host-cell responses. *Cell Mol Life Sci* **68**: 3687-3697.

Stack, J.H., DeWald, D.B., Takegawa, K., and Emr, S.D. (1995) Vesicle-mediated

Protein Transport: Regulatory Interactions between the Vpsl5 Protein Kinase and the Vps34 PtdIns 3-Kinase Essential for Protein Sorting to the Vacuole in Yeast. *J Cell Biol* **129**: 321-334.

Stack, J.H., Herman, P.K., Schu, P.V., and Emr, S.D. (1993) A membrane-associated complex containing the Vpsl 5 protein kinase and the Vps34 Pi 3-kinase is essential for protein sorting to the yeast lysosome-like vacuole. *EMBO J* **12**: 2195-2204.

Steele-Mortimer, O. (2008) The *Salmonella*-containing vacuole: moving with the times. *Curr Opin Microbiol* **11**: 38-45.

Stefan, C.J., Audhya, A., and Emr, S.D. (2002) The yeast synaptojanin-like proteins control the cellular distribution of phosphatidylinositol (4,5)-bisphosphate. *Mol Biol Cell* **13**: 542-557.

Stirling, F.R., and Evans, T.J. (2006) Effects of the type III secreted pseudomonal toxin ExoS in the yeast *Saccharomyces cerevisiae*. *Microbiology* **152**: 2273-2285.

Strahl, T., Hama, H., DeWald, D.B., and Thorner, J. (2005) Yeast phosphatidylinositol 4-kinase, Pik1, has essential roles at the Golgi and in the nucleus. *J Cell Biol* **171**: 967-979.

Strahl, T., and Thorner, J. (2007) Synthesis and function of membrane phosphoinositides in budding yeast, *Saccharomyces cerevisiae*. *Biochim Biophys Acta* **1771**: 353-404.

Stroupe, C., Collins, K.M., Fratti, R.F., and Wickner, W. (2006) Purification of active HOPS complex reveals its affinities for phosphoinositides and the SNARE Vam7p. *EMBO J* **25**: 1579–1589.

Suh, B.C., and Hille, B. (2008) PIP2 is a necessary cofactor for ion channel function: how and why? *Annu Rev Biophys* **37**: 175-195.

Sun, Y., Kaksonen, M., Madden, D.T., and Drubin, D.G. (2005) Interaction of Sla2p's ANTH Domain with PtdIns(4,5)P2 Is Important for Actin-dependent Endocytic Internalization. *Molecular Biology of the Cell* **16**: 717-730

Szentpetery, Z., Varnai, P., and Balla, T. (2010) Acute manipulation of Golgi phosphoinositides to assess their importance in cellular trafficking and signaling. *Proc Natl Acad Sci U S A* **107**: 8225-8230.

Tahirovic, S., Schorr, M., and Mayinger, P. (2005) Regulation of intracellular phosphatidylinositol-4-phosphate by the Sac1 lipid phosphatase. *Traffic* **6**: 116-130.

Takatori, S., Tatematsu, T., Cheng, J., Matsumoto, J., Akano, T., and Fujimoto, T. (2016) Phosphatidylinositol 3,5-Bisphosphate-Rich Membrane Domains in Endosomes and Lysosomes. *Traffic* **17**: 154-167.

Tindall, B.J., Grimont, P.A., Garrity, G.M., and Euzeby, J.P. (2005) Nomenclature and

taxonomy of the genus *Salmonella*. *Int J Syst Evol Microbiol* **55**: 521-524.

Tong, J., Manik, M.K., Yang, H., and Im, Y.J. (2016) Structural insights into nonvesicular lipid transport by the oxysterol binding protein homologue family. *Biochim Biophys Acta* **1861**: 928-939.

Townes, J.M. (2010) Reactive arthritis after enteric infections in the United States: the problem of definition. *Clin Infect Dis* **50**: 247-254.

Tucker, C.L. (2002) High-throughput cell-based assays in yeast. *drug discovery today* **7**: S125-S130.

Uchiya, K.i., Groisman, E.A., and Nikai, T. (2004) Involvement of *Salmonella* Pathogenicity Island 2 in the Up-Regulation of Interleukin-10 Expression in Macrophages: Role of Protein Kinase A Signal Pathway. *Infect Immun* **72**: 1964-1973.

Uchiya, K., and Nikai, T. (2008) *Salmonella* virulence factor SpiC is involved in expression of flagellin protein and mediates activation of the signal transduction pathways in macrophages. *Microbiology* **154**: 3491-3502.

Uchiya, K., Sugita, A., and Nikai, T. (2009) Involvement of SPI-2-encoded SpiC in flagellum synthesis in *Salmonella* enterica serovar Typhimurium. *BMC Microbiol* **9**: 179.

Urbanus, M.L., Quaile, A.T., Stogios, P.J., Morar, M., Rao, C., Di Leo, R., Evdokimova, E., Lam, M., Oatway, C., Cuff, M.E., Osipiuk, J., Michalska, K., Nocek, B.P., Taipale, M., Savchenko, A., and Ensminger, A.W. (2016) Diverse mechanisms of meta effector activity in an intracellular bacterial pathogen, Legionella pneumophila. *Mol Syst Biol* **12**: 893.

Ustun, S., Muller, P., Palmisano, R., Hensel, M., and Bornke, F. (2012) SseF, a type III effector protein from the mammalian pathogen *Salmonella* enterica, requires resistance-gene-mediated signalling to activate cell death in the model plant Nicotiana benthamiana. *New Phytol* **194**: 1046-1060.

Van Engelenburg, S.B., and Palmer, A.E. (2010) Imaging type-III secretion reveals dynamics and spatial segregation of *Salmonella* effectors. *Nat Methods* **7**: 325-330.

Vevea, J.D., Swayne, T.C., Boldogh, I.R., and Pon, L.A. (2014) Inheritance of the fittest mitochondria in yeast. *Trends Cell Biol* **24**: 53-60.

Vinh, D.B.N., Ko, D.C., and Miller, S.I. (2010) Expression of the *Salmonella* Spp. Virulence Factor SifA in Yeast Alters Rho1 Activity on Peroxisomes. *Mol Biol of Cell* **21**: 3567–3577.

Vonaesch, P., Sellin, M.E., Cardini, S., Singh, V., Barthel, M., and Hardt, W.D. (2014) The *Salmonella* Typhimurium effector protein SopE transiently localizes to the early SCV and contributes to intracellular replication. *Cell Microbiol* **16**: 1723-1735.

Wada, Y., Sun-Wada, G.H., Tabata, H., and Kawamura, N. (2008) Vacuolar-type proton ATPase as regulator of membrane dynamics in multicellular organisms. *J Bioenerg Biomembr* **40**: 53-57..

Wang, K., Yang, Z., Liu, X., Mao, K., Nair, U., and Klionsky, D.J. (2012) Phosphatidylinositol 4-kinases are required for autophagic membrane trafficking. *J Biol Chem* **287**: 37964-37972.

Wang, L., Seeley, E.S., Wickner, W., and Merz, A.J. (2002) Vacuole fusion at a ring of vertex docking sites leaves membrane fragments within the organelle. *cell* **108**: 357–369.

Weber, S.S., Ragaz, C., and Hilbi, H. (2009a) The inositol polyphosphate 5-phosphatase OCRL1 restricts intracellular growth of Legionella, localizes to the replicative vacuole and binds to the bacterial effector LpnE. *Cell Microbiol* **11**: 442-460.

Weber, S.S., Ragaz, C., and Hilbi, H. (2009b) Pathogen trafficking pathways and host phosphoinositide metabolism. *Mol Microbiol* **71**: 1341-1352.

Weber, S.S., Ragaz, C., Reus, K., Nyfeler, Y., and Hilbi, H. (2006) Legionella pneumophila exploits PI(4)P to anchor secreted effector proteins to the replicative vacuole. *PLoS Pathog* **2**: e46.

Wickner, W. (2010) Membrane fusion: five lipids, four SNAREs, three chaperones, two nucleotides, and a Rab, all dancing in a ring on yeast vacuoles. *Annu Rev Cell Dev Biol* **26**: 115-136.

Worley, M.J., Nieman, G.S., Geddes, K., and Heffron, F. (2006) *Salmonella* typhimurium disseminates within its host by manipulating the motility of infected cells. *Proc Natl Acad Sci U S A* **103**: 17915-17920.

Wu, H., Jones, R.M., and Neish, A.S. (2012) The *Salmonella* effector AvrA mediates bacterial intracellular survival during infection in vivo. *Cell Microbiol* **14**: 28-39.

Wu, X., Chi, R.J., Baskin, J.M., Lucast, L., Burd, C.G., De Camilli, P., and Reinisch, K.M. (2014) Structural insights into assembly and regulation of the plasma membrane phosphatidylinositol 4-kinase complex. *Dev Cell* **28**: 19-29.

Wurmser, A.E., and Emr, S.D. (1998) Phosphoinositide signaling and turnover: PtdIns(3)P, a regulator of membrane traffic, is transported to the vacuole and degraded by a process that requires lumenal vacuolar hydrolase activities. *EMBO* **17(17):**4930-42.

Yang, Z., Soderholm, A., Lung, T.W., Giogha, C., Hill, M.M., Brown, N.F., Hartland, E., and Teasdale, R.D. (2015) SseK3 Is a *Salmonella* Effector That Binds TRIM32 and Modulates the Host's NF-kappaB Signalling Activity. *PLoS One* **10**: e0138529.

Yip, C.K., Kimbrough, T.G., Felise, H.B., Vuckovic, M., Thomas, N.A., Pfuetzner, R.A., Frey, E.A., Finlay, B.B., Miller, S.I., and Strynadka, N.C. (2005) Structural characterization of the molecular platform for type III secretion system assembly.

Nature **435**: 702-707.

Yoshida, S., Ohya, Y., Goebl, M., Nakano, A., and Anraku, Y. (1994a) A Novel Gene, STT4, Encodes a Phosphatidylinositol 4-Kinase in the PKCl Protein Kinase Pathway of *Saccharomyces* cerevisiae. *J Biochem* **269**.

Yoshida, S., Ohya, Y., Nakano, A., and Anraku , Y. (1994b) Genetic interactions among genes involved in the STT4-PKC1 pathway of *Saccharomyces* cerevisiae. *Molecular and general genetics* **242**: 631-640

Yu, X.J., Ruiz-Albert, J., Unsworth, K.E., Garvis, S., Liu, M., and and Holden, D.W. (2002) SpiC is required for secretion of Salmonella Pathogenicity Island 2 type III secretion system proteins. *Cell Microbiol* **4(8)**: 531-540.

Yu, X.J., Liu, M., and Holden, D.W. (2016) *Salmonella* Effectors SseF and SseG Interact with Mammalian Protein ACBD3 (GCP60) To Anchor *Salmonella*-Containing Vacuoles at the Golgi Network. *MBio* **7**.

Yue, M., Han, X., De Masi, L., Zhu, C., Ma, X., Zhang, J., Wu, R., Schmieder, R., Kaushik, R.S., Fraser, G.P., Zhao, S., McDermott, P.F., Weill, F.X., Mainil, J.G., Arze, C., Fricke, W.F., Edwards, R.A., Brisson, D., Zhang, N.R., Rankin, S.C., and Schifferli, D.M. (2015) Allelic variation contributes to bacterial host specificity. *Nat Commun* **6**: 8754.

Schutz, M., Schöppe, J., Sedlák, E., Hillenbrand, M., Nagy- Davidescu, G., Ehrenmann, J., Klenk, C., Egloff, P., Kummer, L., and Plückthun, A. (2016) Directed evolution of G protein-coupled receptors in yeast for higher functional production in eukaryotic expression hosts. *Sci Rep* **6**:21508

Zhang, X., and Mosser, D.M. (2008) Macrophage activation by endogenous danger signals. *J Pathol* **214**: 161-178.

Zhang, Y., Higashide, W.M., McCormick, B.A., Chen, J., and Zhou, D. (2006) The inflammation-associated *Salmonella* SopA is a HECT-like E3 ubiquitin ligase. *Mol Microbiol* **62**: 786-793.

Zhao, W., Moest, T., Zhao, Y., Guilhon, A.A., Buffat, C., Gorvel, J.P., and Meresse, S. (2015) The *Salmonella* effector protein SifA plays a dual role in virulence. *Sci Rep* **5**: 12979.

APPENDIX

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The *Salmonella* effector SteA binds phosphatidylinositol 4-phosphate for subcellular targeting within host cells

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Summary

Many bacterial pathogens use specialized secretion systems to deliver virulence effector proteins into eukaryotic host cells. The function of these effectors depends on their localization within infected cells, but the mechanisms determining subcellular targeting of each effector are mostly elusive. Here, we show that the S*almonella* type III secretion effector SteA binds specifically to phosphatidylinositol 4-phosphate [PI(4)P]. Ectopically expressed SteA localized at the plasma membrane (PM) of eukaryotic cells. However, SteA was displaced from the PM of *Saccharomyces cerevisiae* in mutants unable to synthesize the local pool of PI(4)P and from the PM of HeLa cells after localized depletion of PI(4)P. Moreover, in infected cells, bacterially translocated or ectopically expressed SteA localized at the membrane of the *Salmonella*-containing vacuole (SCV) and to *Salmonella*-induced tubules; using the PI(4)P-binding domain of the *Legionella* type IV secretion effector SidC as probe, we found PI(4)P at the SCV membrane and associated tubules throughout *Salmonella* infection of HeLa cells. Both binding of SteA to PI(4)P and the subcellular localization of ectopically expressed or bacterially translocated SteA were dependent on a lysine residue near the N-terminus of the protein. Overall, this indicates that binding of SteA to PI(4)P is necessary for its localization within host cells.

Introduction

Salmonella enterica serovars are intracellular facultative pathogens with a wide host range and causing serious illnesses in humans. For example, *S. enterica* serovar Typhi causes typhoid fever while serovar Typhimurium is a major cause of gastroenteritis. *S. enterica* virulence is related to its capacity to deliver about 40 bacterial effector proteins into host cells through the *Salmonella* pathogenicity island 1 and the *Salmonella* pathogenicity island 2-encoded type III secretion systems (SPI-1 and SPI-2 T3SSs) (Figueira and Holden, 2012; Ramos-Morales, 2012; LaRock *et al*., 2015). The SPI-1 T3SS mediates the delivery of effectors critical for invasion of non-professional phagocytes, such as epithelial cells (Galán and Curtiss, 1989). Within host cells, the bacterium is enclosed in a membrane-bound compartment, known as *Salmonella*-containing vacuole (SCV), in which the SPI-2 T3SS is activated and mediates the delivery of effectors contributing to bacterial intracellular replication (Cirillo *et al*., 1998; Hensel *et al*., 1998). However, in addition to invasion and intracellular replication, SPI-1 and SPI-2 effectors also modulate other features of the host–*Salmonella* interaction, such as immune signalling (Figueira and Holden, 2012; Ramos-Morales, 2012; LaRock *et al*., 2015). Furthermore, SPI-1 T3SS effectors also contribute to bacterial intracellular replication (Ramos-Morales, 2012; LaRock *et al*., 2015), and some effectors can be translocated into host cells by both the SPI-1 and the SPI-2 T3SSs (Figueira and Holden, 2012; Ramos-Morales, 2012; LaRock *et al*., 2015).

Bacterial effectors exert their function at specific locations within host cells (Hicks and Galán, 2013). In the case of intravacuolar bacterial pathogens, effectors frequently localize at the membrane of the pathogen-containing vacuoles. For example, several *Salmonella* effectors have been detected on the SCV membrane and on different *Salmonella*-induced

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